

PHOTORECEPTOR MEMBRANE TURNOVER

IN INSECTS

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DECLARATION

All the original research described in this thesis is entirely my own with the following exception: in chapter V, David Blest assisted with the interpretations of electron micrographs and wrote a first draft of the text.



David Williams

Chapters II-IX and the appendix are presented as published or submitted for publication. In addition, during the course of this PhD study, I have had the following published or submitted for publication:

Papers

- 1979. Williams DS The feeding behaviour of New Zealand Dolomedes species (Araneae: Pisauridae). N Z J Zool 6:95-105
- 1979. Williams DS The physiological optics of a nocturnal semi-aquatic spider, Dolomedes aquaticus (Pisauridae). Z Naturforsch 34c:463-469
- 1980. Williams DS, McIntyre P The principal eyes of a jumping spider have a telephoto component. Nature 288:578-580
- 1980. Blest AD, Williams DS, Kao L The posterior median eyes of the dinopid spider Menneus. Cell Tissue Res 211:391-403
- 1981. Williams DS A jumping spider's view. Hemisphere 26:178-183

1982. Blest AD, Hardie RC, McIntyre P, Williams DS The spectral sensitivities of identified receptors and the function of retinal tiering in the principal eyes of a jumping spider. J Comp Physiol A 145:227-239

Abstracts

1980. Williams DS, McIntyre P A deep fovea enhances visual acuity in animals with small heads. Proc Austr Physiol Pharmacol Soc 11:182P
1981. Williams DS Daily changes in rhabdom structure and function in a locust. Proc Austr Physiol Pharmacol Soc 12:80P

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Charles Darwin

(in a letter to Asa Gray, February 1860)

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SUMMARY

1. The compound eyes of three insects were examined in relation to aspects of photoreceptor membrane turnover.

2. At dawn the ommatidial structure of the compound eye of the tipulid fly, Ptilogyna, undergoes the following changes, which are reversed at dusk: (a) the primary pigment cells move into a more central position, immediately distal to the rhabdom, forming a very narrow field-stop; (b) the rhabdom migrates proximally away from the base of the cone; (c) the cross-sectional area and the length of the rhabdomeres begin to diminish. The rhabdom consists of regions with different organisations: the most distal region is closed; just proximal to this region it is open with an intraommatidial extracellular space to which all rhabdomeres are exposed; in the proximal two-thirds it is partitioned by processes from the retinular cells so that the individual rhabdomeres are contained within "pockets". The rhabdomeres twist along their length.

3. During fixation for electron microscopy, myelin-like whorls develop from dipteran photoreceptive microvilli when calcium ions are added to buffer solutions of primary aldehyde fixatives and primary fixation is longer than a few hours. Membrane that is about to be shed appears to be the most likely to form whorls. Whorl-formation can be prevented by including EGTA to chelate the calcium in the buffer solutions.

4. In Ptilogyna, the bulk of the photoreceptor membrane is shed during turnover by abscission from the distal ends of the microvilli into extracellular space. Shed membrane is phagocytosed from the extracellular space by the retinular cells. It then accumulates as vesicles

within multivesicular bodies, and is degraded through multilamellar bodies to residual bodies. Morphological evidence suggesting a similar extracellular route of shedding was found in the compound eye of the more advanced dipteran, the blowfly, Lucilia.

5. In Lucilia, the cross-sectional areas of the rhabdomeres remain constant on a daily cycle or even after periods of prolonged light or darkness.

6. The rhabdoms in the compound eye of Locusta vary in size as a result of daily turnover: shedding reduces their cross-sectional area by 4- to 5-fold at dawn, while assembly of new microvillar membrane increases it by a corresponding amount at dusk. The size of the ommatidial field-stop, produced by the primary pigment cells, changes in tandem with rhabdom size. The density of intramembrane particles on the P-face of the microvillar membrane, exposed by freeze-fracture and putatively representing mostly rhodopsin molecules, does not change.

7. In locusts, shedding and assembly are initiated by the onset of light and darkness respectively, but are also under some degree of endogenous control. They can be initiated within individual ommatidia by local states of illumination, leaving neighbouring ommatidia unaffected.

8. Intracellular recordings of locust photoreceptors measured the increase in angular acceptance and sensitivity to an extended source that is effected by the increase in the size of the field-stop and rhabdom. No change was detected in spectral sensitivity, sensitivity to a point source on-axis, and bump latency between the large and small rhabdom and field-stop states.

CHAPTER I

GENERAL INTRODUCTION

Insects account for about 75% of all the described species of animals. Within this abundance is an extreme diversity of form and habits. Some are very beautiful, others are simply horrid. They inhabit most terrestrial and freshwater environments: from deserts to rainforests, from mountains to the seashore. They are phytophagous, carnivorous, parasitic, or saprophagous.

The major visual organ of adult insects is a pair of compound eyes. In some, particularly the parasitic forms, these eyes are reduced or absent. By contrast, the compound eyes of the fast-flying dragonflies or male hoverflies occupy nearly the entire head.

Compound eyes are also the dominant type of eye in crustaceans; but most other animals, such as ourselves, see solely with what are known, often rather misleadingly, as "simple eyes". The fundamental difference between compound and simple eyes is one of optical design. Whereas all the photoreceptors of simple eyes share the same lens or lens system, in a compound eye small lenslets overlies groups of usually 8 or 9 photoreceptor cells. Each group of photoreceptors, with their lenslet, and cone and pigment cells, constitutes an ommatidium.

There are two basic types of compound eye: superposition eyes in which light may be gathered from many lenslets on to one photoreceptor, and apposition eyes (Fig. 1) in which each ommatidium remains optically isolated. The majority of insects have apposition eyes. Light from a small part of the environment enters a lenslet of an apposition eye and is directed on to the rhabdom (Fig. 1), which is composed of numerous photoreceptive microvilli that project from the

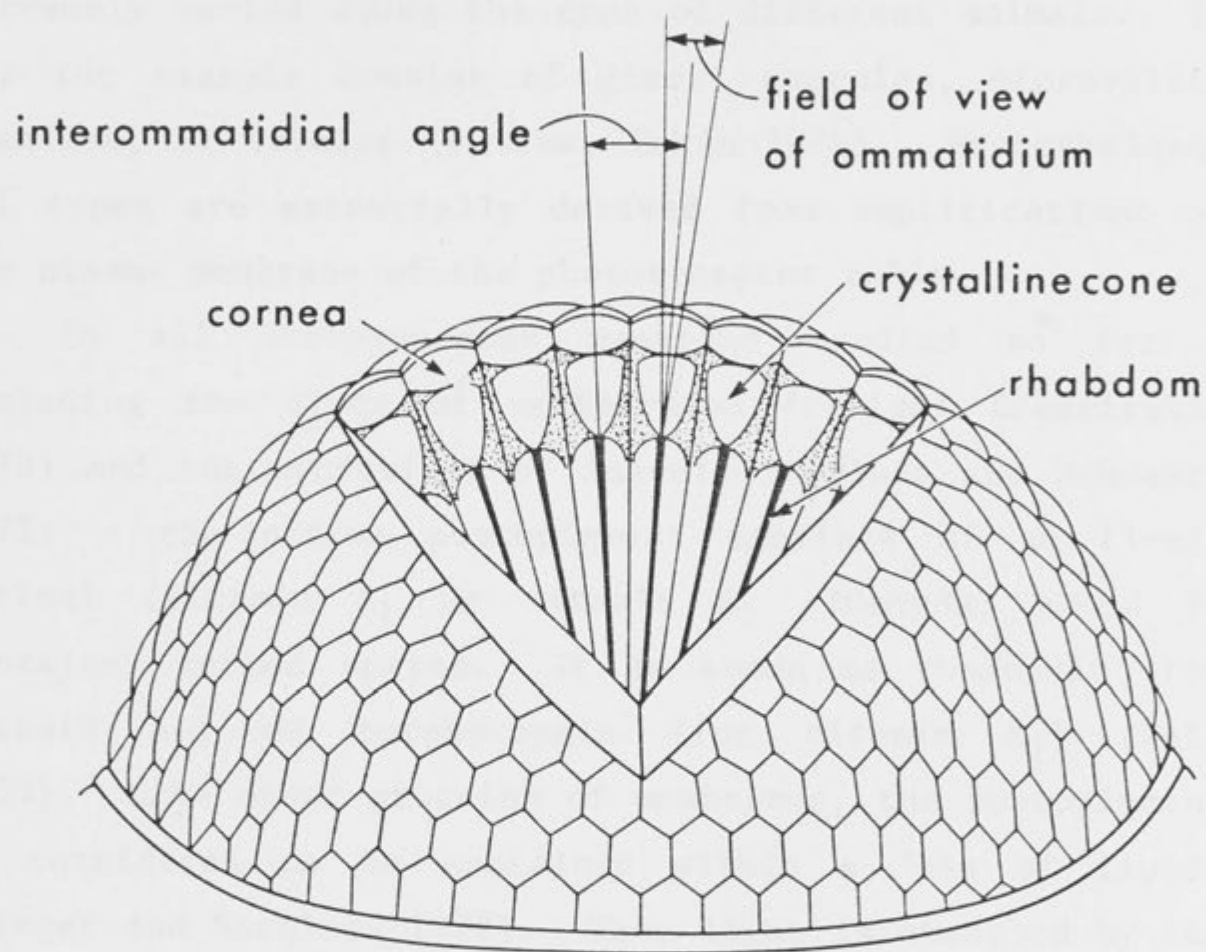


Fig. 1. Schematic representation of an apposition compound eye, cut away to show the ommatidia (modified from Horridge 1977).

photoreceptor cells. The angles between ommatidia and over which the rhabdom accepts light are important factors in determining the eye's acuity. The latter is affected by the optics of the lenslet, and the position and exposed area of the distal tip of the rhabdom.

The arrangement of photoreceptor membrane is extremely varied among the eyes of different animals. It may for example consist of discs, saccules, microvilli, lamellae, or tubules (review: Eakin 1972). Nevertheless, all types are essentially derived from amplifications of the plasma membrane of the photoreceptor cells.

In all photoreceptor membrane studied so far - including the discs of vertebrates (review: Crescitelli 1972) and the microvilli of insects (Paulsen and Schwemer 1972) - the native photopigment consists of an 11-cis retinal (vitamin A₁ or vitamin A₂ aldehyde) bound to proteins, termed opsins. It is known as rhodopsin (for vitamin A₁) or porphyropsin (for vitamin A₂) (Wald 1953). Like other proteins of membranes, the photopigment is considered to be contained within a "sea of lipid" (Singer and Nicolson 1972). When light is absorbed by the photopigment, a change in the sodium conductance of the photoreceptor cell yields an electrical response, which is transmitted to higher-order neurons.

About 15 years ago, initial reports were published showing that photoreceptor membrane turns over; i.e. it is renewed. These findings were consistent with the developing general concept that nearly every component of an organism must be renewed within a given space of time. (The major exception is the DNA of the nucleus.) Sherrington (1940) wrote that "We are no longer in that age, when the comment of the learned on Roger Bacon's famous woman of Norwich who fasted for twenty years, was

that to fast is no more marvellous than to need to eat. We have to be renewed, . . .". In some parts of organisms, replacement is achieved mainly by turning over whole cells (e.g. the red blood cells and epithelial cells of gut villi). In others, the cells per se remain, but their components turn over. Extracellular materials (e.g. connective tissues) and circulating macromolecules (e.g. blood proteins) also turn over (review: Holtzman 1976). Awareness that cellular components are renewed arose from pioneering tracer studies with radioactive isotopes (Hevesey 1962; Schoenheimer and Rittenberg 1940; Leblond and Walker 1956) and development of the autoradiographic technique (Leblond 1965). The necessity of turnover may stem partly from the fact that macromolecules are subject to spontaneous changes; hence it would seem advantageous for proper functioning of a cell that these molecules be continuously replaced (Novikoff and Holtzman 1976).

This thesis examines some aspects of the turnover of photoreceptor membrane in insects. To provide a background for these studies, some of the published work on the turnover of membranes in general, and the turnover of photoreceptor membrane of vertebrates and arthropods is briefly discussed.

Turnover of Membranes

Eukaryotic cells have an extensive membrane system, of which the larger part includes the plasma membrane, the Golgi apparatus, the endoplasmic reticulum (ER), and the nuclear, mitochondrial (and chloroplast) membranes (Palade 1975). Further membrane is found in lysosomes and other vesicular structures. The cycling of this membrane has been studied primarily in cells where it occurs in conjunction with the secretion of substances into the

extracellular space, or the uptake of substances from it. Examples of the first include the presynaptic terminals of neurons and the secretory cells of glands such as the anterior pituitary, thyroid, pancreas, lactating mammary gland, and the salt gland of marine reptiles and birds. At times, rate of secretion from these cells can be very high, demanding a rapid cycling of membrane. Substances for secretion are packaged in the Golgi by membrane originating from the ER. The resulting membrane-delimited vacuoles then migrate to the plasma membrane, fuse with it, and release their contents into the extracellular space. Membrane is returned to the interior from the plasma membrane by vesicles that pinch off from it. Fibroblasts are an example of cells in which the uptake of substances has been studied. These cells are widespread in connective tissues, and are readily grown in culture. They selectively take up several macromolecules, including low-density lipoprotein (Goldstein et al. 1979), epidermal growth factor (Gordon et al. 1978), α_2 -macroglobulin (Willingham et al. 1979), and insulin (Maxfield et al. 1978), in endocytotic vesicles.

In many instances, the pathway of secretion or adsorptive pinocytosis involves vesicles that are coated with a basket-like network, consisting mainly of the protein, clathrin (Kanaseki and Kadota 1969; Pearse 1978; for beautiful illustrations from deeply-etched freeze-fracture replicas, see Heuser and Evans 1980). Examples include the endocytotic vesicles in fibroblasts (Goldstein et al. 1979) and presynaptic terminals of neurons (Heuser and Evans 1980), and the vesicles that bud from the Golgi apparatus in the secretory cells of the pancreas and parotid salivary gland (Jamieson and Palade 1971; Castle

et al. 1972) and the exporting cells of the lactating mammary gland (Franke et al. 1976).

Currently, much interest concerns the fate of the endocytosed plasma membrane. There are two alternatives: either the membrane is broken down into small molecules (lysed) (Holtzman 1976), or it is reused without undergoing lysis and resynthesis de novo (Palade 1959, 1975). Both seem to occur. Tracer studies show two separate pathways for the retrieval of the luminal membrane in the rat pancreas (Herzog and Reggio 1980) and lacrimal and parotid glands (Herzog and Farquhar 1977), the surface membrane of the anterior pituitary (Farquhar 1978), and the luminal membrane of thyroid follicles removed during the endocytosis of thyroglobulin (Herzog and Miller 1979): one route leads exclusively to lysosomes, the other to Golgi cisternae, where the membrane appears to be reused for packaging the secretion product. In neurons, new synaptic vesicle membrane is manufactured "near the Golgi apparatus" in the perikaryon (and perhaps by the smooth ER in the axon), and retrieved presynaptic membrane can apparently be degraded via multivesicular bodies (review: Holtzman and Mercurio 1980). However, within this pathway membrane is extensively reused to package more transmitter (Heuser and Reese 1973; Heuser 1978): pulse-labelling shows the half-lives of synaptic vesicles to be several weeks, so that reasonable estimates of neurotransmission rates indicate a vesicle reuse of thousands to millions of times (Holtzman et al. 1977). The situation is less clear in the fibroblasts. When low-density lipoprotein is taken up, it is transferred to lysosomes, and the receptor molecules seem to be immediately returned to the plasma membrane (Anderson et al. 1976); but the fate of the vesicular

membrane is unknown (Pearse and Bretscher 1981).

Turnover of Photoreceptor Membrane in Vertebrates

The turnover of vertebrate photoreceptor membrane was first convincingly shown by the autoradiographic studies of Young (1967) and Young and Bok (1969). Earlier workers had shown that the protein component of rod outer segments was renewed (Droz 1963), and suggested that "myeloid bodies" in the pigment epithelium might be detached portions of outer segments engulfed by the pigment epithelium (Bairati and Orzalesi 1963; Feeney et al. 1965). However, Young and Bok were able to follow the sequence of events involved in turnover by examining the utilisation of radioactive amino acids in the rods of frogs. The labelled amino acids were first incorporated into the inner segment, and subsequently accumulated at the base of the outer segment, from whence they migrated along the outer segment as an intensely-labelled band (Young 1967). After this band disappeared from the end of the outer segment (6 weeks later), highly radioactive inclusion bodies appeared in the overlying pigment epithelium (Young and Bok 1969). Young (1967) proposed that it was not only protein that was displaced along the outer segment and eventually taken up by the pigment epithelium, but that the entire disc structure was turned over in this manner (Fig. 2a).

Turnover of cone outer segments was not so readily recognised, because the labelled proteins are scattered diffusely among the discs (Young 1969). This observation led Young (1971) to propose that there was no bulk turnover of cone photoreceptor membrane, and that instead it was possibly renewed by molecular replacement. Morphological studies that showed phagocytosis of the

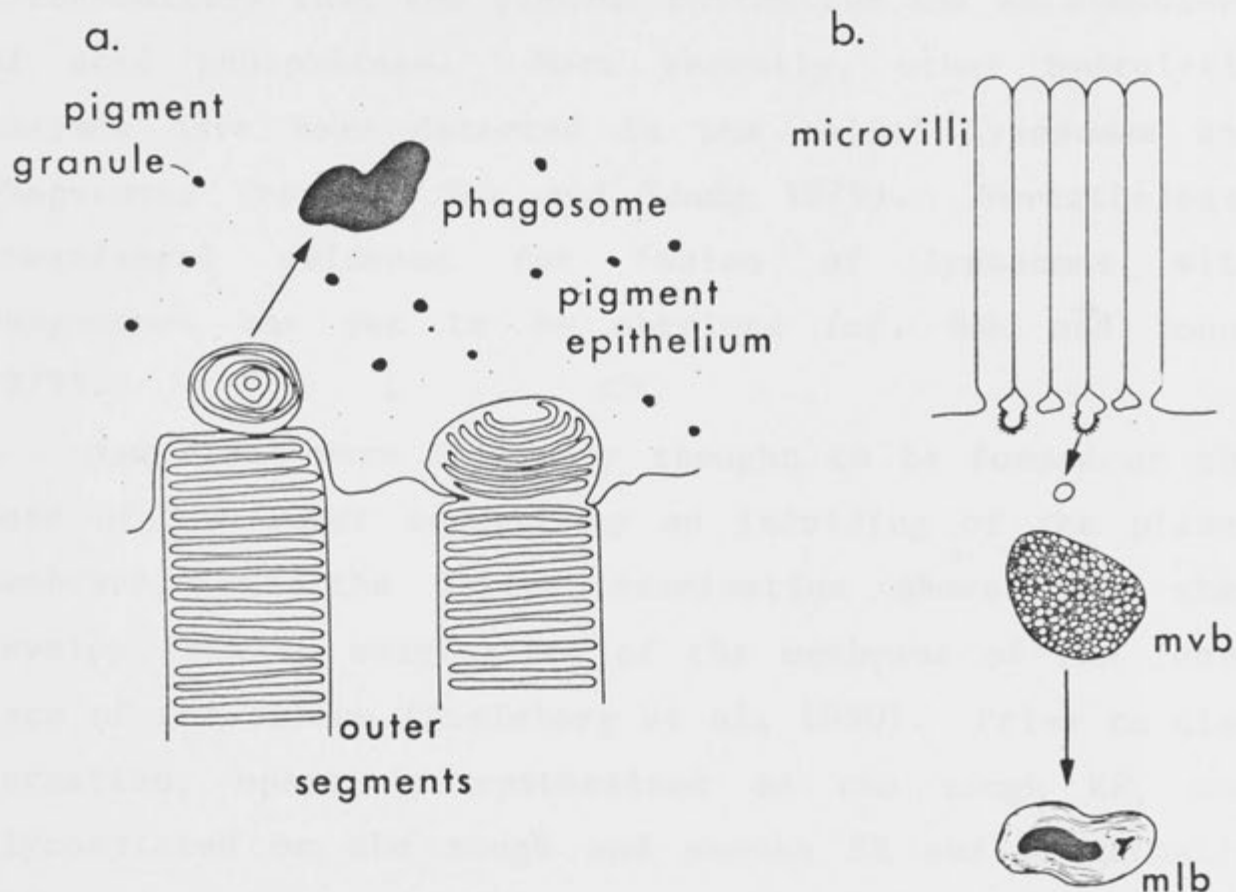


Fig. 2. The common means of disposal of photoreceptor membrane during turnover in vertebrates and arthropods. a in vertebrates the terminal discs of the cone or rod outer segments are phagocytosed by the pigment epithelium. b in arthropods the microvillar membrane pinches off into the photoreceptor cell and is collected in multivesicular bodies (mvbs), which degrade to multilamellar bodies (mlbs).

terminal discs of cones in man (Hogan et al. 1974) and squirrels (Anderson and Fisher 1975, 1976) soon refuted this hypothesis.

After phagocytosis by the pigment epithelium, the shed package of discs, or "phagosome", is considered to be degraded. Eichner (1955, 1958) first showed by cytochemistry that the pigment epithelium has an abundance of acid phosphatase. More recently, other hydrolytic enzymes have been detected in the cells' lysosomes and phagosomes (review: Bok and Young 1979). Nevertheless, unequivocal evidence for fusion of lysosomes with phagosomes has yet to be obtained (cf. Bok and Young 1979).

New discs were initially thought to be formed at the base of the outer segment by an infolding of the plasma membrane, but the latest examination shows that they develop from an evagination of the membrane of the inner face of the cilium (Steinberg et al. 1980). Prior to disc formation, opsin is synthesised on the rough ER, and glycosylated on the rough and smooth ER and (terminally with trimming) on the Golgi apparatus (Young 1974; Bok et al. 1977; Papermaster et al. 1978). Short chains of mannose and N-acetylglucosamine, attached to its NH_2 -terminal regions, account for most of rhodopsin's carbohydrate (Fukuda et al. 1979). Once synthesised, the opsin glycoprotein remains associated with membrane (Bok et al. 1977). Transport to the inner ciliary membrane is therefore considered to proceed in preformed membrane packages, but the mechanisms of delivery are still much a matter of conjecture. As a working hypothesis, it is widely assumed that vesicles of preformed membrane fuse with the cell surface (Papermaster et al. 1975; Kinney and Fisher 1978; Besharse 1980; Besharse and Pfenninger 1980),

and therefore insert membrane into it in a manner comparable to exocytosis during secretion in gland cells and nerve terminals (see above). Components incorporated into the surface membrane and destined for disc membrane could move along the plasma membrane to the point of evagination into discs. The chromophore appears to be added to opsin after it reaches the outer segment (Bok et al. 1977).

The turnover of discs occurs on a daily cycle. The shedding of rod outer segments peaks at dawn (LaVail 1976; Basinger et al. 1976), while the shedding of cone outer segments is restricted to just after dusk (O'Day and Young 1978) or at least the night-time (Tabor et al. 1980). New rod discs are added primarily during the first 8 hours of the day (Besharse et al. 1977a).

The control of these cyclic changes has been a topic of recent interest. The first studies showed a major difference between shedding in rats and frogs: in rats light was not necessary for normal shedding (LaVail 1976), whereas in frogs it was (Basinger et al. 1976). Shedding in rats has since been shown to have a true circadian nature (LaVail 1980). There is also an element of endogenous control in amphibians, although light is the more influential factor (Besharse et al. 1977b). Attempts to localise the control mechanisms of turnover have shown that they are effective in each eye independent of the other. Shedding in the frog, Rana, is prevented if one eye is masked at dawn without affecting shedding in the unmasked eye (Hollyfield and Basinger 1978). Initiation of shedding, as well as synchronisation of its circadian timing by the central nervous system, occurs unilaterally in rat eyes (Tierstein et al. 1980). Lastly, in vitro isolation of single eyes does not prevent light-stimulated

shedding and assembly of photoreceptor membrane in Xenopus (Flannery and Fisher 1979). Whether or not the control mechanisms can be localised further, to within discrete parts of the retina, is not known. Likewise, the physiological nature of the mechanisms remains a topic for further investigation.

Turnover of Photoreceptor Membrane in Arthropods

The first indications that the rhabdoms of arthropod eyes are not static structures came from Debaisieux (1944), working on several crustaceans, and Sato et al. (1957), who worked with the adult mosquito, Culex. These authors found that the rhabdoms of these animals grew under dark adaptation and diminished under light adaptation. Their findings were corroborated later by electron microscopists, studying the retinal morphology of a wide variety of animals (e.g. White 1967; for references to more recent literature see Williams 1982, or chapter VII). White (1964, 1968) and others (Eguchi and Waterman 1967, 1968; Tuurala and Lehtinen 1967) also observed an increase in the number of multivesicular bodies (mvbs) and multilamellar bodies (mlbs) (cf. Fig. 2) in mosquito or crab photoreceptors when light-adapted. Eguchi and Waterman (1967) suggested that the mvbs were derived from coated vesicles observed around the rhabdom, and the mlbs from mvbs. They proposed that the lamellar bodies "serve as a temporary depot for breakdown products of the biochemical system in vision". Using ferritin as a tracer, White (1968) confirmed that mvbs were fed by coated vesicles that pinched off from the bases of the rhabdomeric microvilli. With this result, and the knowledge that rhabdom size decreased on exposure to light, he concluded that: "The mvb system thus appears to

be a segment of a cycle of rhabdomeric loss and renewal." Renewal was supposed to occur in order to counterbalance membrane loss; it is clearly of major importance when the rhabdom grows under dark adaptation. Loss of rhabdomeric membrane to mvbs and subsequent states of degradation was later considered to be equivalent to the phagocytosis and lysis of outer segment discs in vertebrates (White and Lord 1975) (Fig. 2).

The next major aspect of photoreceptor membrane turnover to be disclosed in arthropods was that it occurs on a daily cycle, as it does in vertebrates. Blest (1978) showed that when the spider, Dinopis, was kept on a natural light cycle, some 90% of the microvillar membrane is shed in the 2 hours after dawn and collects in mvbs before being broken down. At the onset of night, rhabdom size is restored as new membrane is assembled. The occurrence of a variation in rhabdom size between day and night in many arthropods indicates that a daily turnover pattern of a similar kind is quite common. In some, shedding appears to peak at dawn and cease at night, while assembly of new membrane is possibly continuous (e.g. mosquito, White and Lord 1975, Brammer et al. 1978; White et al. 1980; crayfish, Hafner et al. 1980). Autoradiographic evidence indicates that Limulus rhabdoms behave in this manner (Krauchs et al. 1976); however, Chamberlain and Barlow (1979) suggest an alternative scheme, which Stowe (1981) disputes. Others are more comparable to Dinopis: massive shedding and assembly both occur during short discrete periods of the daily cycle (e.g. crab, Stowe 1980; locust, Williams 1982, or chapter VII). The discovery of this cycle has clearly facilitated experiments on the mechanisms of shedding and assembly: done at the appropriate time of day, a normal and maximal

response is guaranteed. Moreover, the presence of the daily cycle immediately poses the question of how it is controlled. Animals in which the shedding and assembly phases of turnover are completely separated are particularly well-suited as subjects for studies of the mechanisms of control.

Shedding of Photoreceptor Membrane. Since White (1968), the mechanisms involved in photoreceptor membrane shedding have been explored further. Firstly, freeze-fracture studies have confirmed that primary endocytosis from microvilli is followed by secondary endocytosis of coated vesicles as they enter the smooth membranous envelope of an mvb (Eguchi and Waterman 1976). Secondly, a series of cytochemical studies have described the lysis of the products of shedding. At dawn in Dinopis an atypical GERL system, which differentiates from rough ER, injects hydrolytic enzymes into secondary lysosomes that are usually at a later stage than mvbs and mlbs (Blest et al. 1978a, b; Blest et al. 1979). Somewhat differently, in crab photoreceptors acid phosphatase is found in early mvbs and comes from both Golgi bodies and ER (Blest et al. 1980). Shed photoreceptor membrane in arthropods (and vertebrates) does not appear to be recycled in the same way as the luminal membrane of secretory cells and the presynaptic membrane of neurons. Thirdly, the way in which membrane is actually discarded from the rhabdom has been shown to vary according to how the rhabdom is organised. Pinocytosis from the bases of the microvilli is not universal. Instead, disposal via an extracellular route occurs in some arthropods (Blest and Maples 1979; Williams and Blest 1980, or chapter V).

Assembly of New Photoreceptor Membrane. As in vertebrates, discovery of the mechanisms of assembly lags somewhat behind our understanding of the processes of shedding and subsequent catabolism. Morphological evidence so far suggests that ER is the immediate precursor of microvilli (Itaya 1976; Whittle 1976; Blest and Day 1978; Stowe 1980, 1981); that is, the Golgi apparatus does not appear to play the same important synthetic role as it does in most other systems that require the manufacture of glycoproteins (cf. Farquhar and Palade 1981). Unfortunately, autoradiographic studies of arthropod retinæ, unlike those of vertebrate retinæ (Young and Droz 1968; Bok et al. 1977), have been unsuccessful in attempts to label organelles along the synthetic pathway (cf. Perrelet 1972).

Control. The early studies (above) showed that turnover was influenced by light and darkness. More recently, endogenous effects have also been found (Blest 1978; Chamberlain and Barlow 1979; Nassel and Waterman 1979; Stowe 1981; Williams 1982, or chapter VII). In Limulus, efferent nervous input has been identified as a source of circadian control, for which each eye appears to have its own oscillator (Barlow and Chamberlain 1979). Light and darkness also appear to affect each eye independently: masking experiments have shown that the initiation of assembly of photoreceptor membrane by the onset of darkness is under unilateral control in the crab, Leptograpsus (Stowe 1981). The first evidence showing that turnover is controlled locally within discrete parts of a single retina is presented in this thesis (chapter VIII).

The Present Study

Three aspects of photoreceptor membrane turnover in the insect compound eye are examined in this thesis:

1. The mechanisms of shedding in relation to organisation of the rhabdom.
2. Changes of photoreceptor function caused by or related to the daily pattern of turnover.
3. The control of turnover.

The subjects of the study were the compound eyes of the tipulid fly, Ptilogyna (Fig. 3), the blowfly, Lucilia (Fig. 4), and the locusts, Locusta and Valanga (Fig. 5).

Ptilogyna is one of the largest tipulid flies. In Australia, as elsewhere, there are more species of Tipulidae than any other dipteran family (Colless and McAlpine 1970). They clearly favour damp places, and many are crepuscular. Adult Ptilogyna were collected during the day as they hung from foliage on river banks; their larvae probably inhabit the moist soil of the banks.

Two species of the metallic-coloured genus of blowflies, Lucilia, are found in Australia. The eyes of both were studied. L. cuprina initiates about 80% of the cutaneous myiasis, or "blowfly strike", of sheep; the female oviposits on a sheep's back, where her larvae feed on serous exudates and living tissues. L. sericata causes strikes in some other countries, but is virtually innocuous in Australia (Colless and McAlpine 1970).

Plagues of Locusta migratoria are well-known in the Old World for their devastation of enormous areas of crops. This locust occurs in Australia but rarely swarms (Key 1970). The other locust used, Valanga irregularis, is one of the largest locusts in the world. It feeds on the leaves of trees and shrubs in the moist regions of Northern Australia.



Fig. 3. The large Australian tipulid Ptilogyna. X 8.



Fig. 4. The blowfly, Lucilia cuprina. X 11.

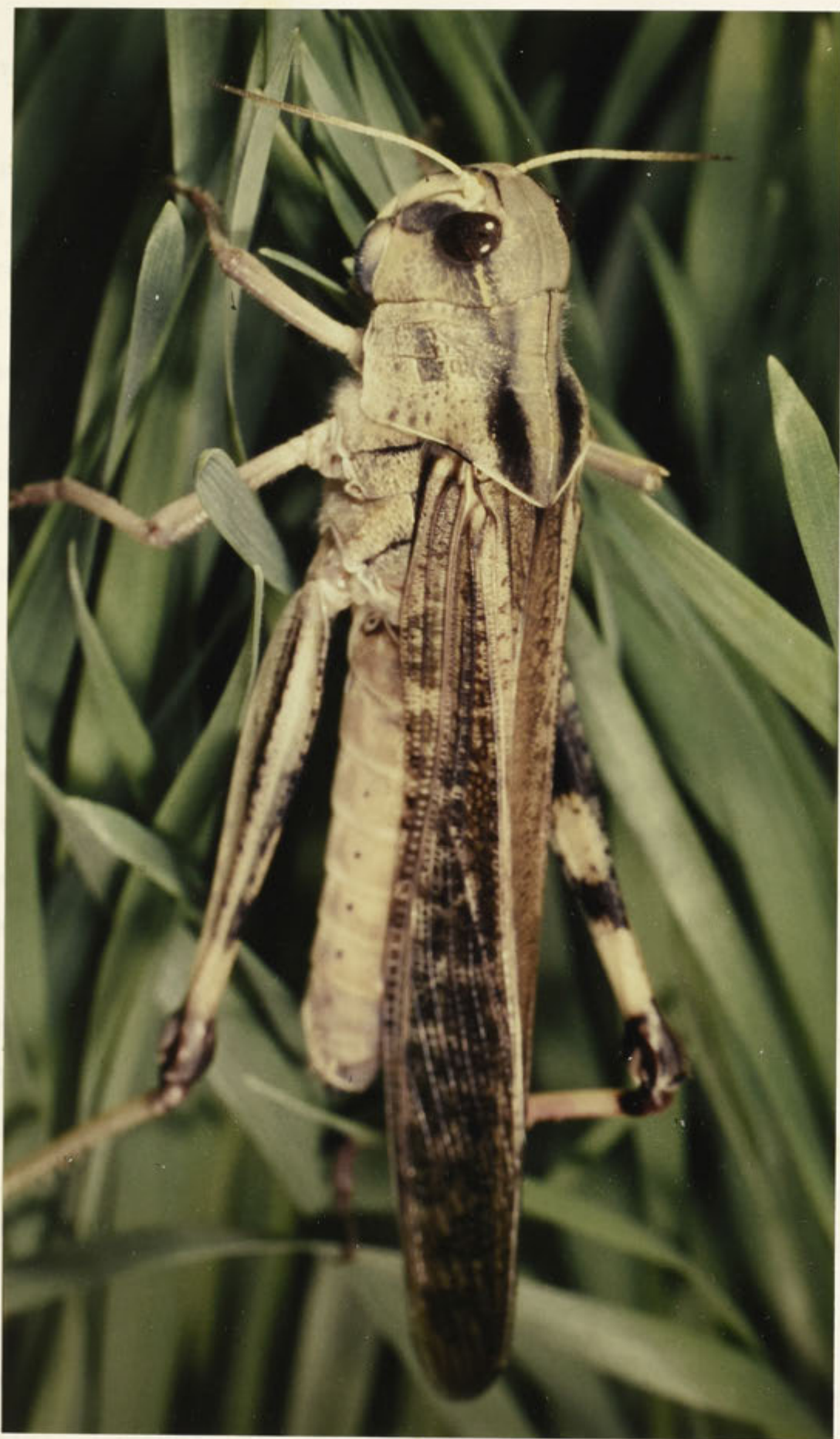


Fig. 5. The locust, Locust migratoria. X 3.

Ptilogyna, Lucilia, and the locusts all have apposition compound eyes. A major difference among them is that the dipterans have open rhabdoms, whereas the locusts, like most other insects, have closed rhabdoms: that is, the rhabdomeres of the flies are separated by extracellular space, while those of locusts are contiguous. (This distinction is discussed further in chapter II.) These insects were chosen because of their suitability to test particular hypotheses (Ptilogyna, locusts), or for their importance to related areas of vision research (Lucilia). Investigations were initiated in response to the following suppositions:

Topic 1. Since the mechanisms of disposal and addition of photoreceptor membrane during turnover are inherently dependent on the arrangement of the receptive segments in relation to adjacent structures, compound eyes with different retinal organisations possibly have different means of turnover. Variations from the common arthropod plan of shedding by pinocytosis from the basal ends of the microvilli had already been found in different spider ocelli (Blest and Day 1978; Blest and Maples 1979). All previous studies on the mechanisms of shedding in compound eyes had been done on eyes with closed rhabdoms; therefore the mechanisms of an eye with an open rhabdom were sought.

A possible means of investigation was by autoradiography. With tritiated leucine as a label, this technique has been used previously to study rhabdom turnover (Burnel et al. 1970; Pepe and Baumann 1972; Perrelet 1972; Krauhs et al. 1976; Hafner and Bok 1977; Stein et al. 1979); however, interpretations from it have proved to be limited (cf. discussion by Perrelet 1972). Labelling of rhabdoms results in no discrete region of

label that moves with time, as occurs in the rod outer segments of vertebrates (cf. Young 1967). Instead a diffuse label is found throughout the rhabdom (Perrelet 1972). A rather diffuse autoradiographic reaction has also been noted among different cell components, obscuring any relationship between the label and specific organelles (Perrelet 1972). The diffuse label of the rhabdom could be caused by a high mobility of newly synthesised proteins within the photoreceptor membrane. However, at least in crayfish microvillar membrane, there is a very limited translational diffusion of proteins (Goldsmith and Wehner 1977). Alternatively, it may result from labelling of artefactually redistributed proteins of the microvillus cytoskeleton (Blest et al. 1982b, or appendix). During conventional fixation, the cytoskeleton is broken down (Blest et al. 1982a), and in tipulid rhabdoms can be shown to be redistributed within each microvillus (Blest et al. 1982b, or appendix). Problems of interpretation of labelling patterns also occur when attempts are made to trace the path taken by microvillar membrane once it is discarded from the rhabdom. Hafner and Bok (1977) found that mvbs in crayfish photoreceptors were labelled 5 minutes after injection of tritiated leucine, and reached a peak density of label after 12 hours. The rise in label density of the mvbs occurred before that of anything else, including the rhabdom, so that it could not have resulted from proteins of membranes that had collected in the mvbs. Hafner and Bok considered that the early radioactivity could represent newly synthesised hydrolytic enzymes introduced into the mvbs. It seemed therefore that if the route of photoreceptor membrane was to be accurately traced, autoradiography, at least with labelled amino acids, would be unsatisfactory.

A more informative means of investigation was considered to be by temporal sampling of the morphology of a rhabdom that sheds daily a large proportion of its membrane in a short space of time. Thus the bulk flow of shed photoreceptor membrane can be followed. This method has an added advantage over autoradiography: it is more straight-forward and versatile. Nevertheless, it necessitated some background investigations.

Firstly, an insect with this type of open rhabdom was found, and the basic morphology of the retinae of its compound eyes examined. The tipulid fly, Ptilogyna, was considered the most suitable; the structure of its compound eyes is described in chapters II and III. Secondly, tests were made for possible artefacts of tissue preparation, so that the true nature of the structural changes during shedding could be determined (chapter IV). (Previously, in a crayfish rhabdom, whorls of membrane that were probably artefactual had been considered to be real structures, analogous to the phagosomes found in the pigment epithelium of the vertebrate retina (Roach and Wiersma 1974).)

On the basis of these findings the mechanism of photoreceptor membrane shedding in Ptilogyna was described from electron microscopy (chapter V). Once the mechanism was elucidated in this primitive dipteran, a comparative argument was used to support less convincing morphological evidence for a similar method of shedding in an advanced dipteran (chapter VI). This description was considered pertinent because the eyes of advance dipterans are virtually a model system in other fields of insect vision research.

Topic 2. Changes in rhabdom size as a result of

photoreceptor membrane turnover are related to light and dark adaptation. A major question asked was: does the change in rhabdom size, or the process of changing its size affect photoreceptor performance either by itself or in conjunction with some other photomechanical change?

With respect to the advanced dipteran rhabdomeres many theoretical studies have predicted that a change in cross-sectional area should alter photoreceptor function. Because of the large body of work on the performance of advanced dipteran photoreceptors, it was considered important to know if rhabdomeral cross-sectional area does in fact vary (chapter VI).

In order to test directly for changes of photoreceptor performance that are related to rhabdom turnover, intracellular recordings of photoreceptor cells at appropriate times of day were made. An animal with variable-size rhabdomeres and photoreceptors from which it was possible to record intracellularly for long periods of time was required. A locust fulfilled this criterion. To provide an anatomical basis for the electrophysiological work, the ommatidial structure of Locusta was analysed in relation to lighting and time of day (chapter VII). The performance of single photoreceptor cells was then monitored in relation to a daily light cycle (chapter IX).

Topic 3. Much can be inferred about the control mechanisms of photoreceptor membrane turnover from morphological changes in the rhabdom. Such changes in a locust were used to examine (1) the nature of the stimuli that initiate the shedding and assembly phases of photoreceptor membrane turnover (chapter VII), and (2) the localisation of the control mechanisms (chapter VIII).

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Organisation of the Compound Eye of a Tipulid Fly During the Day and Night

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Summary. The ultrastructure of the compound eye of the Australian tipulid fly, *Ptilogyna spectabilis*, is described. The ommatidia are of the acone type. The rhabdom corresponds to the basic dipteran pattern with six outer rhabdomeres from reticular cells 1–6 (R1–6) that surround two tiered central rhabdomeres from R7 and 8. Distally, for about 8 μm , the rhabdom is closed. For the remainder, where the rhabdomere of R8 replaces that of R7, the rhabdom is open, and the rhabdomeres lie in a large central ommatidial extracellular space. In the proximal two thirds of the rhabdom, the central space is partitioned by processes from the reticular cells so that the individual rhabdomeres are contained in ‘pockets’.

At night the rhabdom abuts the cone cells, but during the day it migrates some 20 μm proximally and is connected to a narrow (1–2 μm) cone cell tract. This tract is surrounded by two primary pigment cells, which occupy a more lateral position at night and thus act like an iris. Pigment in secondary pigment cells also migrates so as to screen orthodromic light above the rhabdom during the day. Between midday and midnight, the rhabdom changes in length and cross-sectional area as a result of asynchrony of the shedding and synthetic phases of photoreceptor membrane turnover. The effects of these daily adaptive changes on photon capture ability are discussed with regard to the sensitivity of the eye.

A. Introduction

The Tipulidae (crane flies) are a primitive family of Diptera. Previous work on their compound eyes is limited to one study by Sotavalta et al. (1962), who described distal-proximal migrations of the rhabdom and surrounding pigment under dark and light adaptation in five European genera. However, these authors used only light microscopy and consequently could not observe fine structural detail. This paper describes the ultrastructure of the compound eyes of a large crepuscular Australian tipulid, *Ptilogyna spectabilis*, during the day

and at night. It complements a previous account of the method of photoreceptor membrane shedding during turnover in the same fly (Williams and Blest, 1980).

B. Materials and Methods

Adult males and females of the large Australian tipulid (Nematocera, Diptera), *Ptilogyna* (*Plusiomyia*) *gracilis spectabilis* (Skuse) (Dobrotworski, 1971), were collected from the banks of the Mongarlowe River, near Braidwood, New South Wales. They were transported to Canberra in plastic bags in a polystyrene container with a window, and kept in a room exposed to diffuse daylight. Thus, the flies' normal daily light cycle was maintained, and exposure to temperatures beyond the range experienced in the natural environment was prevented (Williams and Blest, 1980).

The compound eyes of flies on a normal daily light cycle were fixed at noon, at midnight, 0.400 h, and at various times around sunrise and sunset. Most of the daily photoreceptor membrane shedding has been shown to be initiated near dawn and completed by midday (Williams and Blest, 1980). Several abnormal regimes were employed: eyes were fixed (1) at 0.800 h, after being held in darkness over dawn; (2) at midnight, after being held under indoor lighting since dusk; and (3) at midnight, after being exposed to 1 h of indoor lighting to interrupt the normal dark period. Indoor lighting was provided by Osram 40 W "daylight" fluorescent tubes. Light reflected off surrounding white walls was about 140 cd/m² (about 350 lux) measured by an Asahi Pentax spot photometer.

The eyes were dissected in primary fixative at 4°C, under light filtered by Schott RG665 (absorbs λ shorter than red) and KG3 (absorbs heat) filters. Room lighting was either on or off to coincide with day or night, or the artificial light regime. The primary fixative was 2.5% glutaraldehyde and 2% paraformaldehyde buffered in 0.1 M sodium dihydrogen orthophosphate-NaOH with 0.07 M D-glucose and 0.9 mM CaCl₂ at pH 7.3 and 4°C. Fixation time varied from 1–24 h; it was not known at the time of the experiments that the longer times caused artefactual whorls to develop at the distal ends of the microvilli in day-adapted samples (see Fig. 6) (Williams, 1980). After a brief wash in buffer, tissues were postfixed for 1–2 h in 1% OsO₄ in the same buffer. They were embedded in Araldite, and silver sections were cut mainly from the central region of the eye with glass knives, and collected on Formvar-coated slot grids. Specimens were examined in a Jeol 100C or Hitachi H500 electron microscope.

C. Results

I. General Arrangement of Ommatidia

The compound eyes of *Ptilogyna spectabilis* are positioned laterally on the head, being well separated by a prominent rostrum. They appear very dark from the exterior, approximately hemispherical but quite small (less than 1.5 mm in diam.). Their strong curvature is even and all facets have a similar diameter, implying little regional specialisation for spatial sampling.

Each facet has a strongly curved corneal surface and diameter slightly greater than 30 μ m. The ommatidia are of the acone type (Grenacher, 1879). Beneath each lenslet are four tractile cone cells, surrounded laterally by two primary pigment cells (PPCs). Proximally the cone cells abut the rhabdom, but also send processes into the rhabdom that are apparent in transverse sections between adjacent retinular cells (Figs. 8, 13, 15). The arrangement of the cone and PPCs and the position of the rhabdom changes between night and day (Fig. 1A and B).

Surrounding the PPCs and retinular cells are numerous secondary pigment cells (SPCs). They extend 100 μ m from the base of the lenslet to the basement

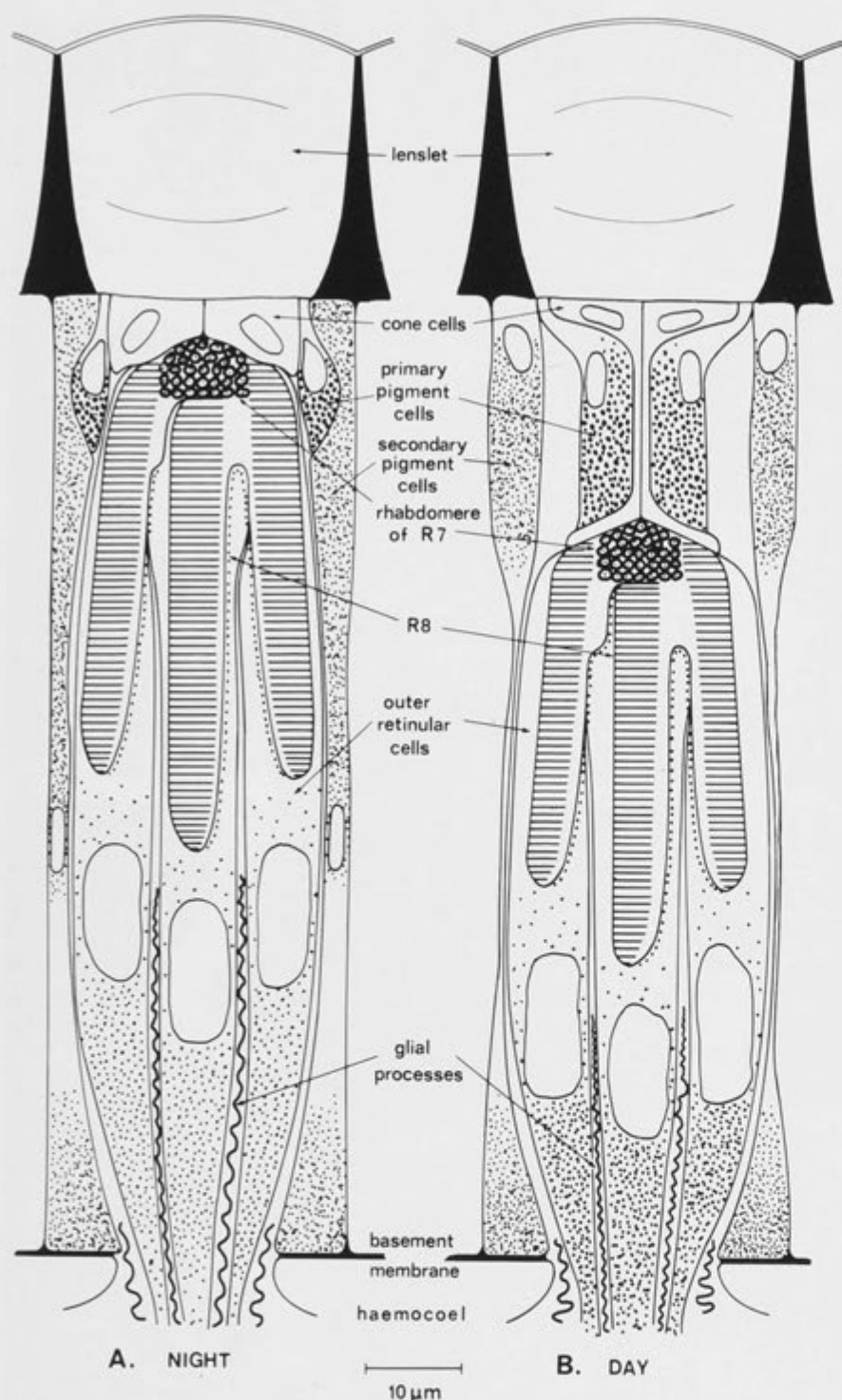


Fig. 1. Schematic longitudinal profiles of night- (A) and day- (B) adapted ommatidia. Nuclei unshaded

membrane. Each ommatidium has 15–18 SPCs in a single ring, but there are further cells outside this layer where the hexagonal ommatidial array allows space (Fig. 3).

II. Retinular Cells

In accordance with the basic dipteran plan (Dietrich, 1909; Trujillo-Cenóz and Melamed, 1966) six retinular cells (R1–6) contribute six outer rhabdomeres

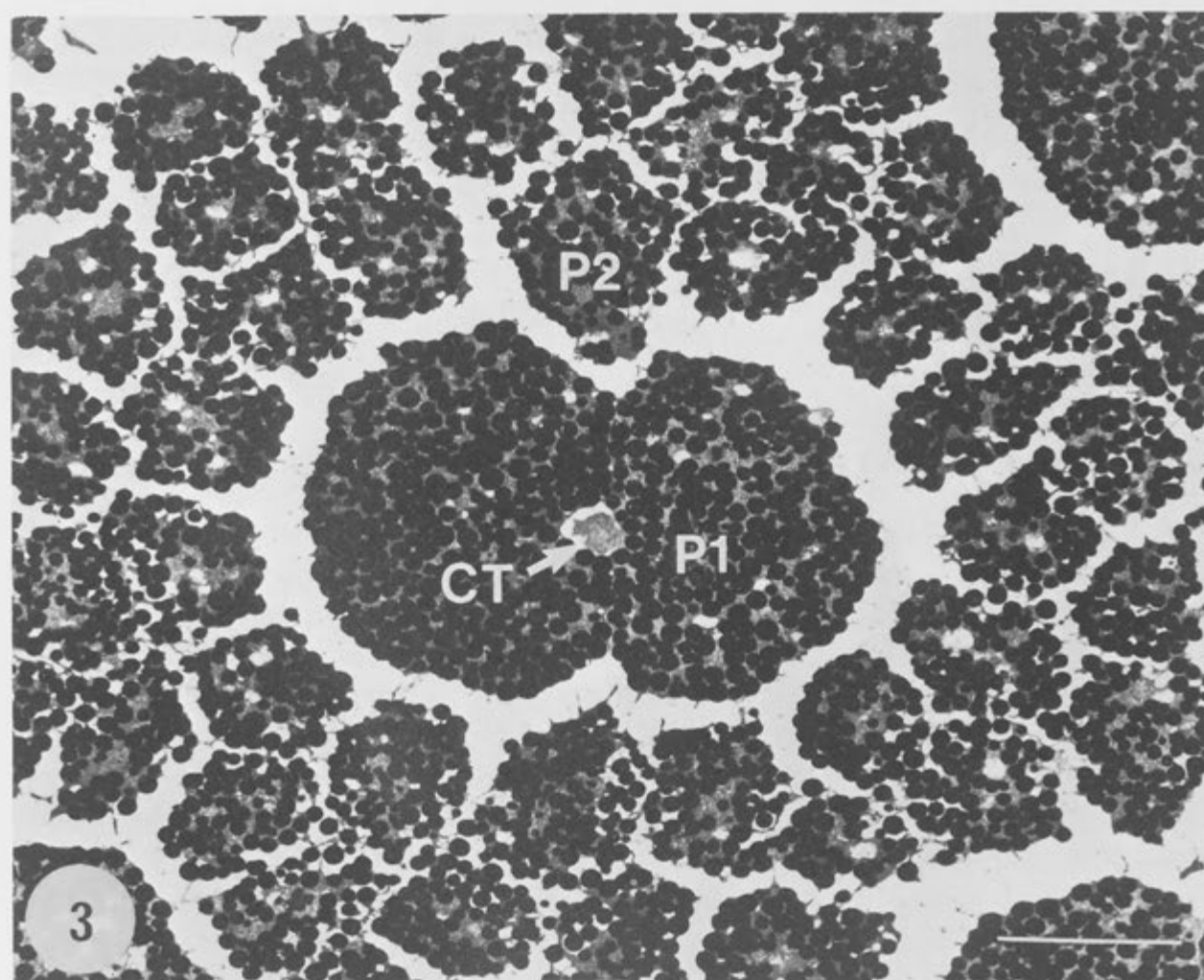
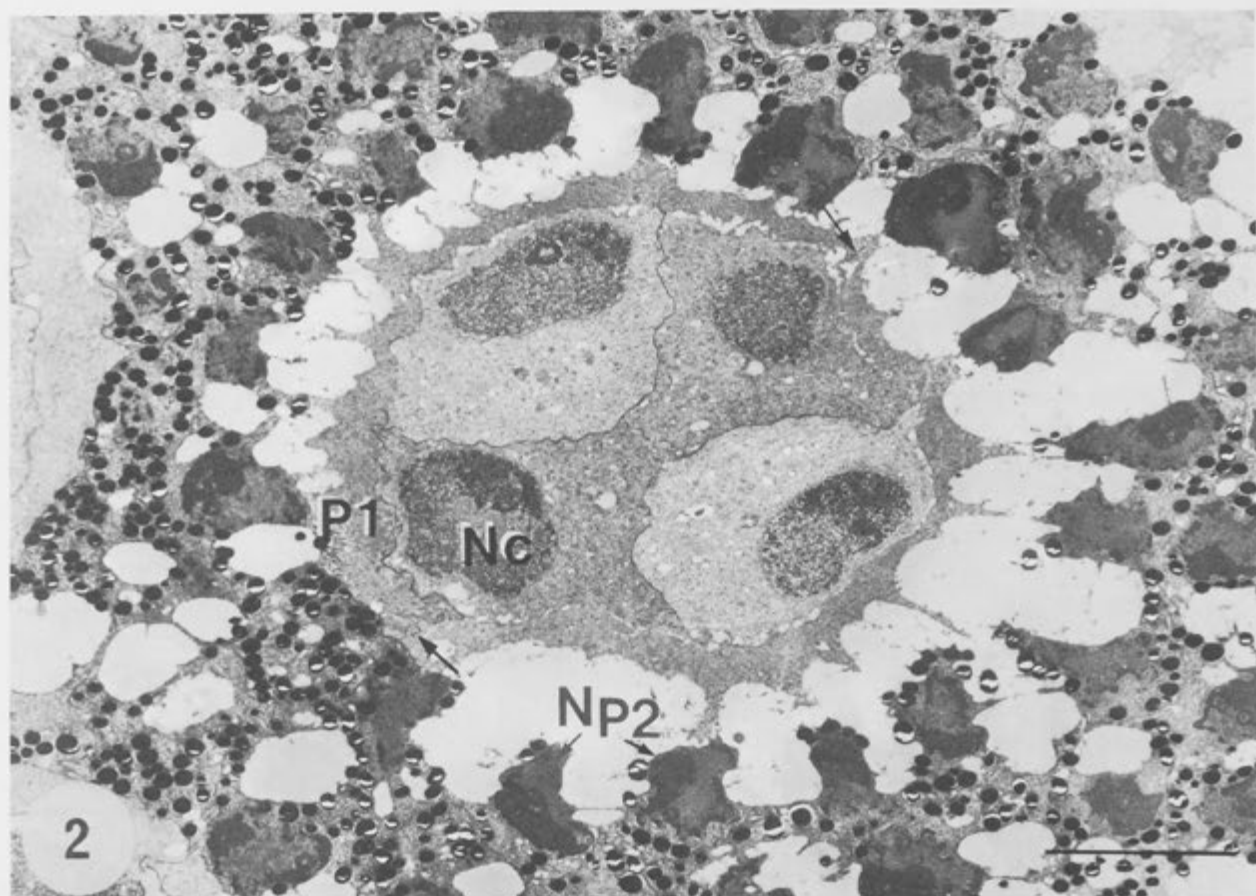


Fig. 2. Transverse section (TS) through cone cell nuclei (N_c) in day-adapted ommatidium. Arrows indicate junctions between primary pigment cells (P_1). (N_{P_2}) nuclei of secondary pigment cells. $\times 3,600$; scale = $5 \mu m$

Fig. 3. TS through cone tract (CT) of day-adapted ommatidium. (P_1 and P_2) primary and secondary pigment cells, respectively. $\times 3,700$; scale = $5 \mu m$

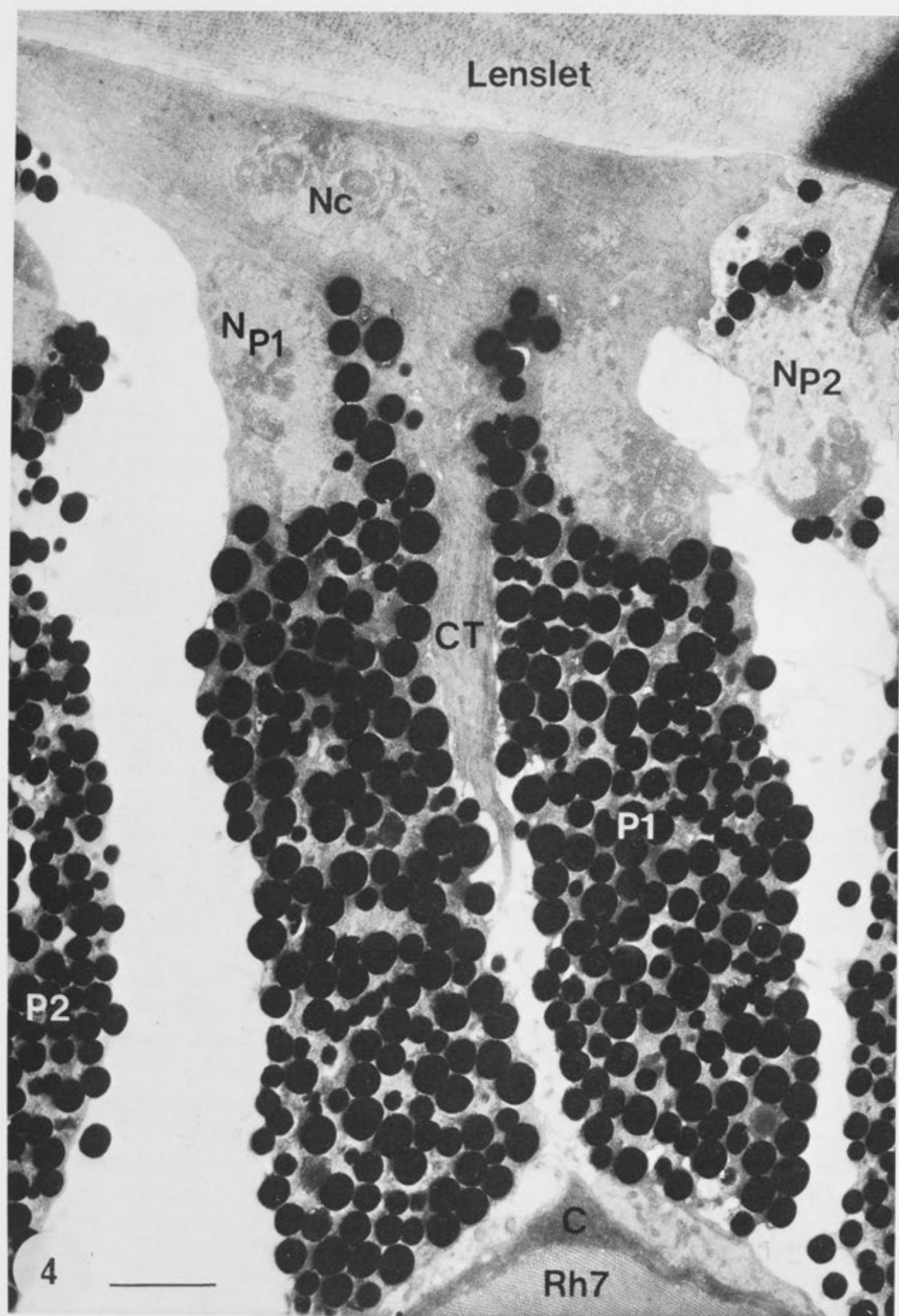


Fig. 4. Near longitudinal section down cone tract (CT). (C) cone; (N_c) cone cell nucleus; (N_{p1} and N_{p2}) primary and secondary pigment cell nuclei, respectively; (P1 and P2) primary and secondary pigment cells; (Rh7) distal part of seventh rhabdomere. $\times 7,300$; scale = 2 μm

that extend the length of the rhabdom, and surround the central rhabdomeres of R7 and 8, which are tiered. But, unlike the retinae of advanced Diptera, the structure of the rhabdom in *Ptilogyna* changes along its length. It may be divided into four regions.

1. For the distal most 8 μm , the central rhabdomere is from R7 and it is opposed to a ring of contiguous rhabdomeres from R1–6 (Figs. 5, 6). In this region, the rhabdom is a continuous mass of microvilli, except for the presence of some cytoplasm of R7, which is limited to a very thin strip along the base of the microvilli and a narrow neck that connects the rhabdomere to its axon outside the perimeter of the rhabdom (Figs. 5, 6).

2. Below the closed region, the rhabdomere of R8 replaces that of R7, and the axon of R7 lies between R1 and 6. For about 7 μm the rhabdom is fully open (Fig. 7), except that directly beneath the closed region, and particularly at night, rhabdomeres were found partially contiguous with their neighbours (Fig. 10). The rhabdomeres lie in a common central extracellular space (ECS) bounded by R1–7 that juxtapose their neighbours and cone cell processes, and at places are joined by desmosomes (Fig. 7).

3. Proximal to region 2, processes from R8 extend around their own rhabdomere, and laterally between the rhabdomeres of R1–6 to the perimeter of the rhabdom, so as to cordon individual rhabdomeres (Fig. 11). The lateral processes meet the descending cone cell processes between adjacent receptor cells, R1–7 (Fig. 13).

4. In a transition zone between regions 3 and 4, the lateral processes of R8 recede as processes from the rhabdomeral margins of R1–6 replace them by developing centrally. Accordingly, each rhabdomere in region 4, the longest and most basal region, is enclosed by its own cell (Fig. 14). The cone cell processes appear in transverse section as elongated strips between R8 and each outer retinular cell, and terminate here (Figs. 14, 15). Regions 3 and 4 include over two thirds of the rhabdom, and may be visualized as having one central and six outer rhabdomeres, each isolated in a 'pocket' with a 'private' ECS that is continuous with the large common central ommatidial ECS in the more distal second region.

Down their length the rhabdomeres of R1–6 move slightly outward from the centre of the ommatidium (Fig. 1), and twist about their central axis (Williams, in preparation). The overall length of the rhabdom is no more than 50 μm , but rhabdomeres may measure more than 5 μm in diam. – twice the entire width of fused rhabdoms found in many other apposition eyes. The rhabdomeres taper abruptly at their proximal ends.

The retinular cell nuclei are contained in a dilated region below the rhabdom (Fig. 16). In this cell body region, the retinular cells are bundled together, and ramifications of a non-pigmented glial cell surround R8 and lie between the outer retinular cells (Fig. 17).

The retinular cells narrow into axons as they pass through the basement membrane, and here the non-pigmented glial supportive system extends to completely envelop R1–7. Hence, below the basement membrane the whole bundle of receptor axons is ensheathed and penetrated by the glia, which appears intimately associated with the retinular cells and contains abundant mitochondria and free ribosomes (Fig. 18).

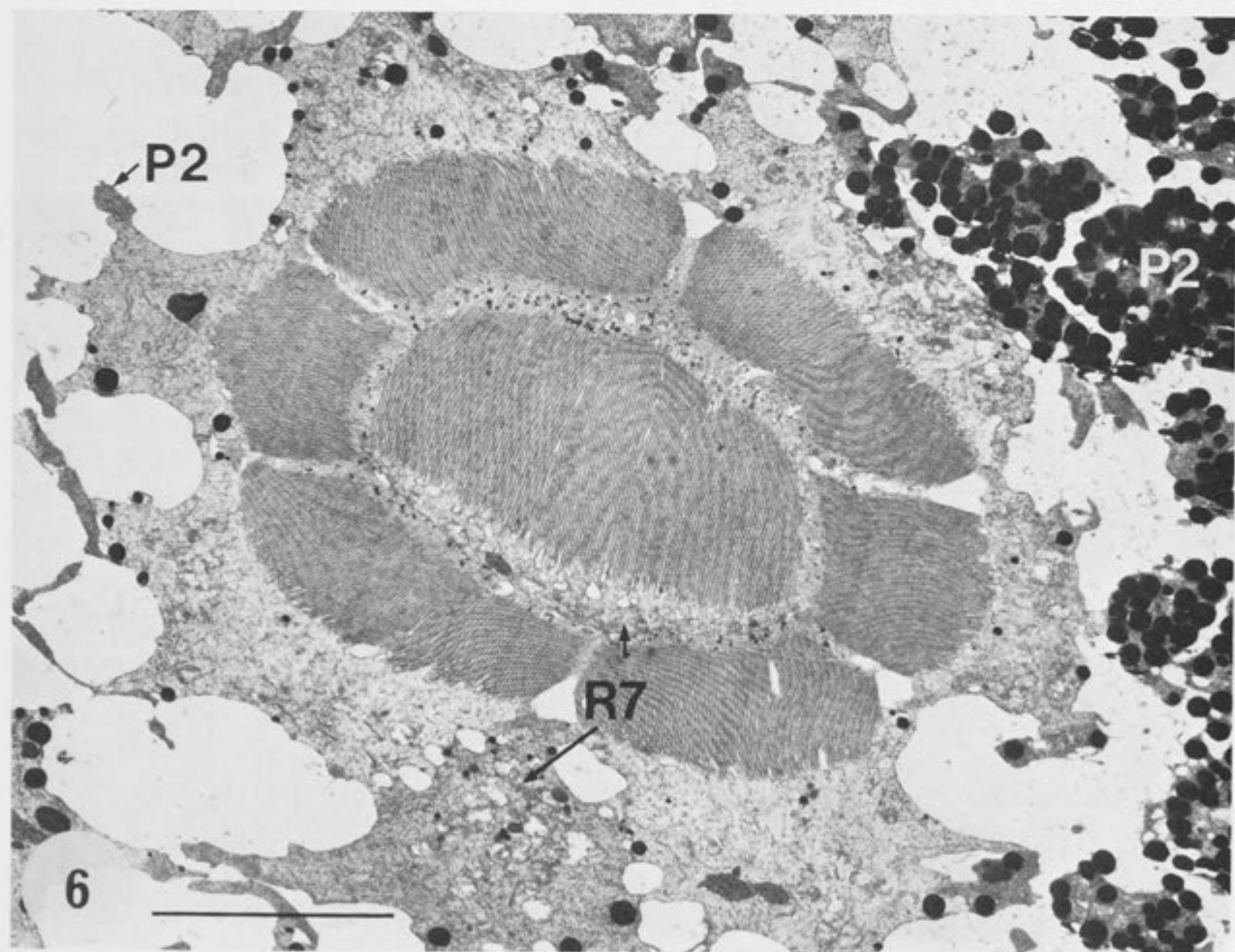
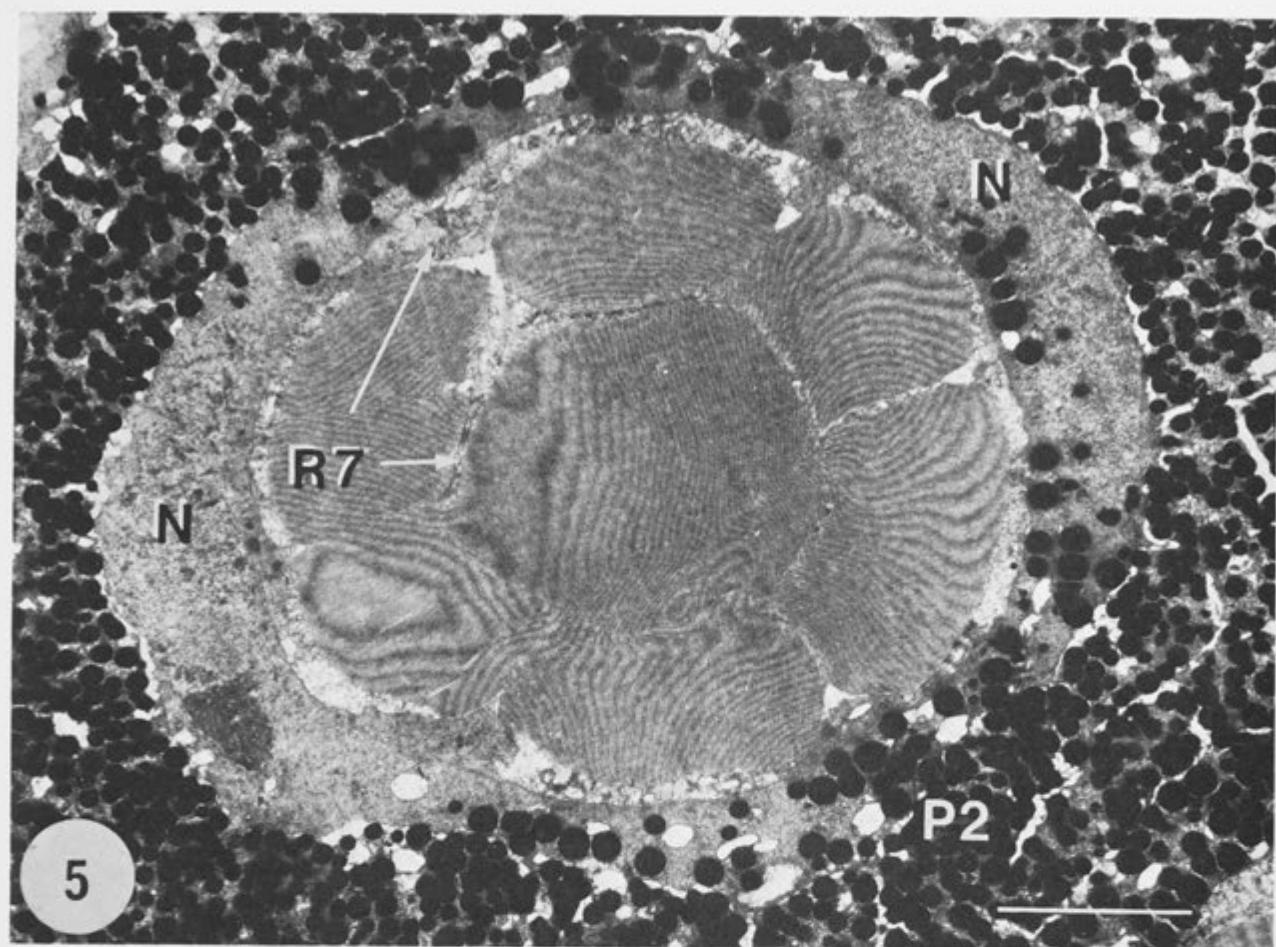


Fig. 5. TS of closed rhabdom region at night. (*N*) nucleus of primary pigment cell; (*P2*) secondary pigment cell; (*R7*) cytoplasm of central retinular cell. $\times 3,900$; scale = $5\ \mu\text{m}$

Fig. 6. Near TS of closed rhabdom region during the day. Right side of section is slightly more distal. (*P2*) secondary pigment cells; (*R7*) cytoplasm of seventh retinular cell. $\times 4,900$; scale = $5\ \mu\text{m}$

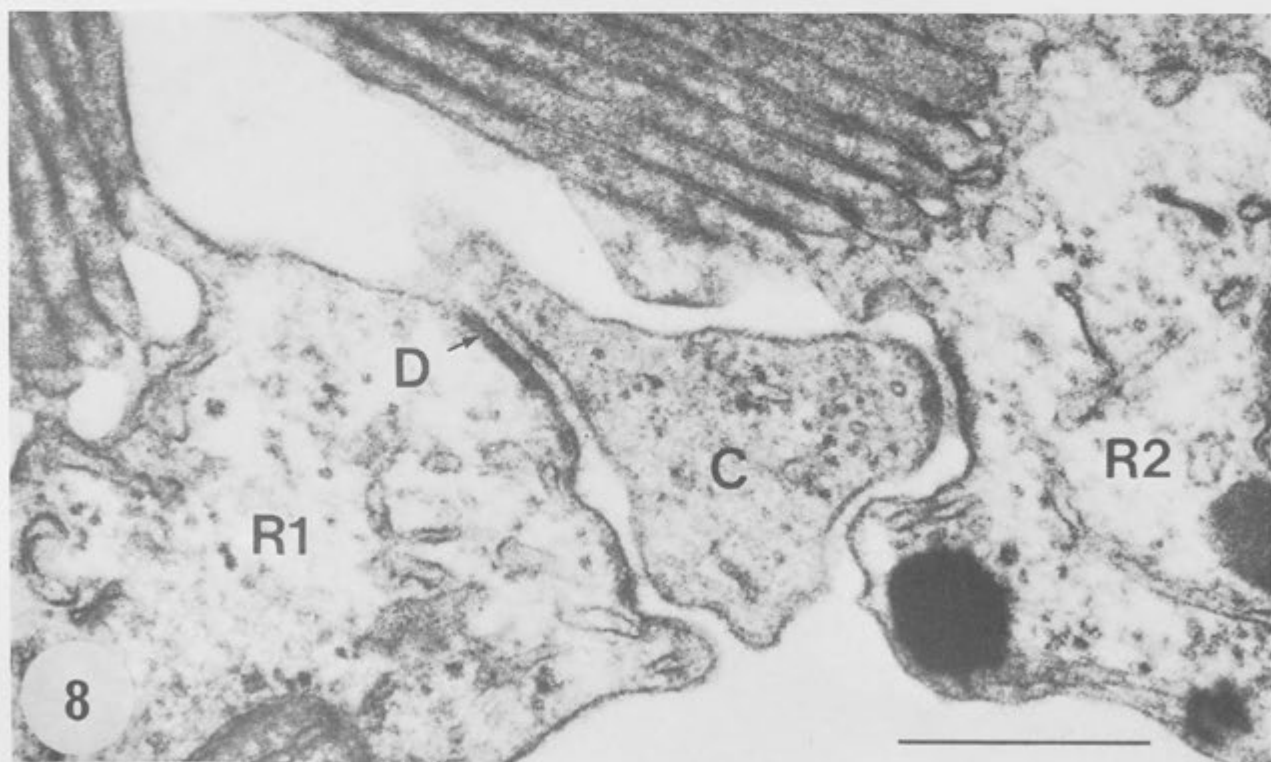
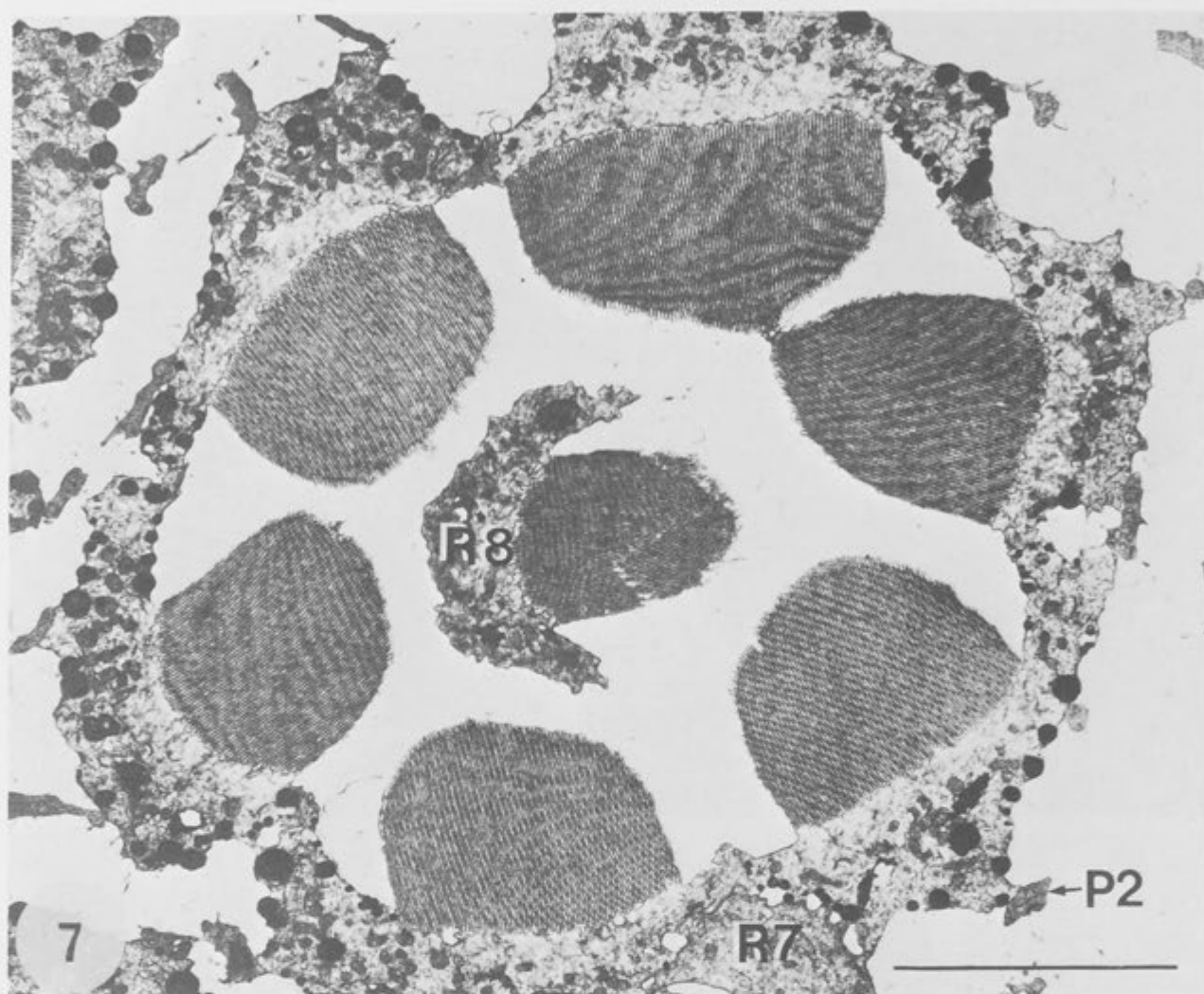


Fig. 7. Second rhabdom region in TS of day-adapted ommatidium. (P2) secondary pigment cells; (R7 and R8) seventh and eighth reticular cells. $\times 5,800$; scale = $5\ \mu\text{m}$

Fig. 8. Cone cell process (C) between two adjacent outer reticular cells (R1, R2) in the same region of the rhabdom as shown in Fig. 7. (D) desmosome. $\times 50,000$; scale = $0.5\ \mu\text{m}$

The first 4 μm of the lamina, through which the ensheathed axons pass, consists mainly of spaces of haemocoel (Fig. 17) that appear similar to those shown to be haemolymph (blood) channels in the locust lamina (Shaw 1978, and personal communication). Beneath these spaces, the glial cells from different axon bundles interconnect to form a continuous network, about 5 μm thick, extending across the lamina (Fig. 17). The nuclei of the glial cells are at this level, one nucleus associated with each axon bundle (Fig. 18).

In the retina, the cytoplasm of the receptor cells is characteristically very dense, with pigment granules of two sizes, ribosomes, both free and attached to endoplasmic reticulum, and abundant mitochondria. In the lamina, the axons appear less dense and contain mitochondria, a few pigment granules, and large numbers of microtubules.

III. Changes Between Night and Day

Rhabdom and Iris. In a dark-adapted ommatidium fixed at night under red light (Fig. 1A), the ends of the rhabdomere lie close to the lens. The layer of cone cells separating the rhabdom from the base of the lenslet is about 3–4 μm deep. Lateral to the cone cells and distal part of the rhabdom is a pigmented annulus formed by the PPCs. After full day adaptation, the rhabdom has migrated some 18–20 μm proximally. The PPCs have closed in above the rhabdom, leaving only an elongated 1–2 μm wide cone tract down the centre to the rhabdom (Figs. 1B, 3, 4). Thus the PPCs appear to function like an iris.

Secondary Pigment Cells and outer Ommatidial Space. At night, the nuclei of the SPCs lie adjacent to the proximal rhabdom. Pigment granules in the SPCs extend from the lenslet down to the level of the cells' nuclei, and are present in the distended most basal 15 μm . They are absent from a zone between these two pigmented regions (Fig. 1A).

During day adaptation, the contents of the SPCs that surround the rhabdom at night migrate distally (Fig. 1). The cells' nuclei come to lie adjacent to the cone cell nuclei (i.e., close to the lenslet), and pigment packs above the level of the rhabdom, except for that in the basal region, which remains unchanged. Around the rhabdom, the SPCs are reduced to narrow columns that contain no obvious structures other than microtubules. They do not isolate adjacent ommatidia as they do at night, and areas between neighbouring rhabdoms consist almost entirely of outer ommatidial ECS that also occupies a significant volume above the rhabdom around the PPCs. This large quantity of ECS in day-adapted eyes is probably not due to hypertonicity of the fixative buffer solutions or too rapid dehydration producing cell shrinkage during tissue processing, because night-adapted material was processed in the same way and exhibited hardly any outer ommatidial ECS anywhere in the retina (compare Figs. 5 and 6, 7 and 10, 11 and 14). Furthermore, intermediate states between full night and day adaptation indicate that the change in the amount of outer ommatidial ECS is part of the normal slow adaptation process.

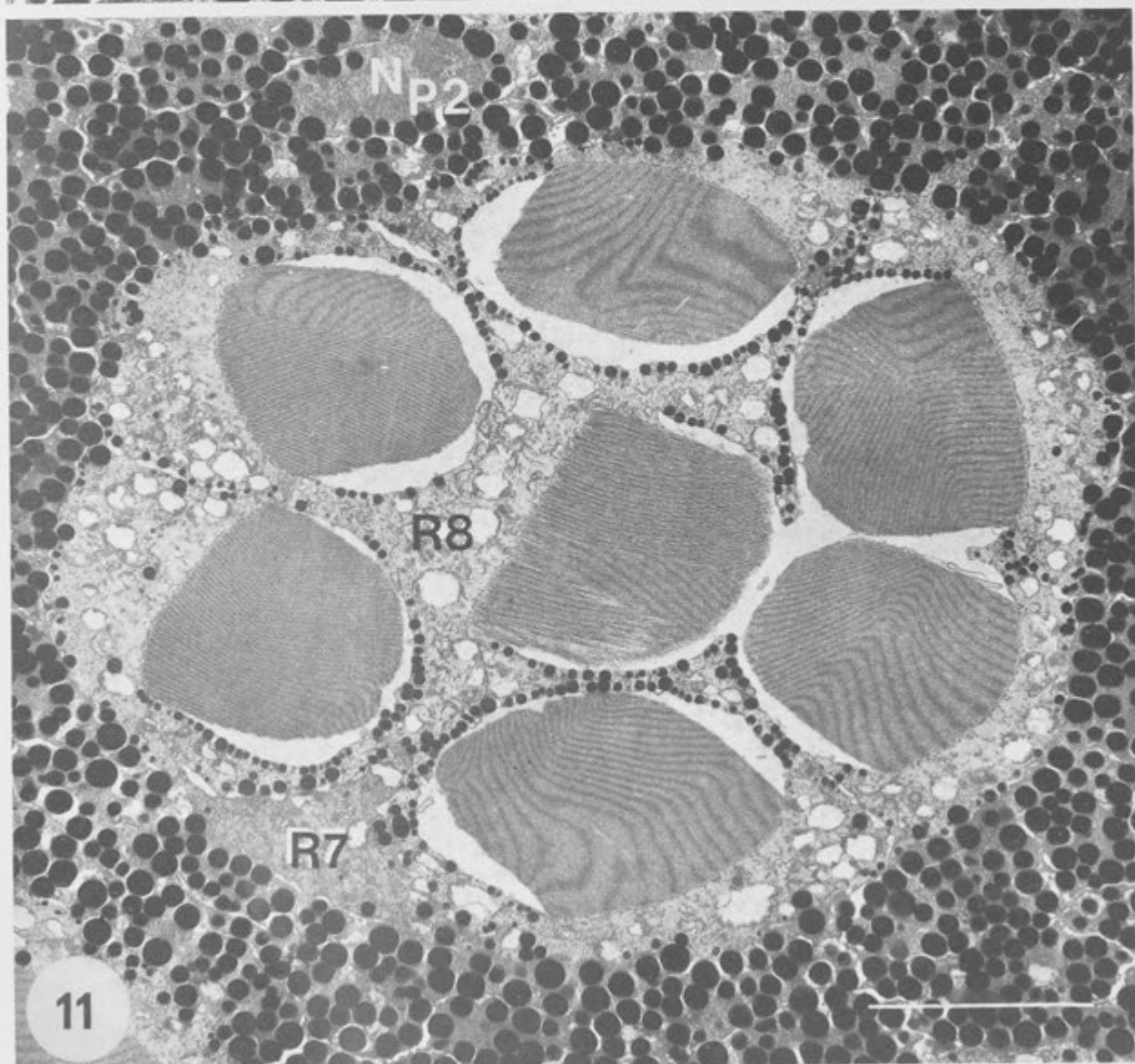
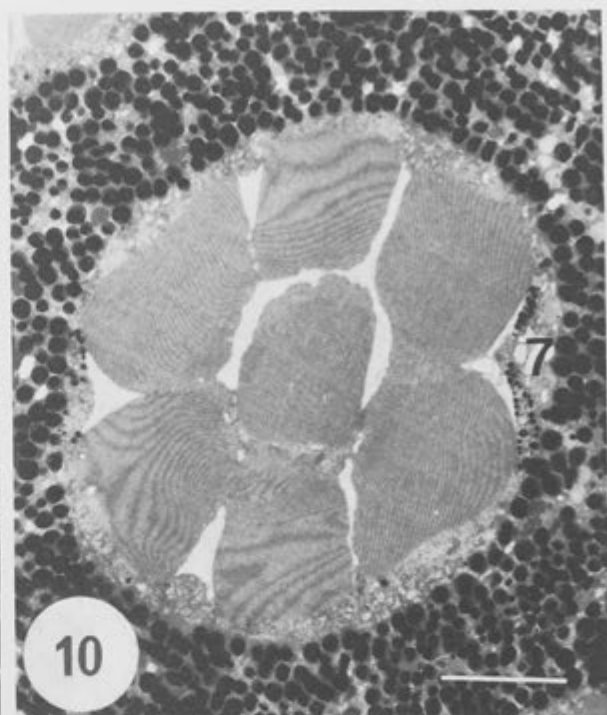
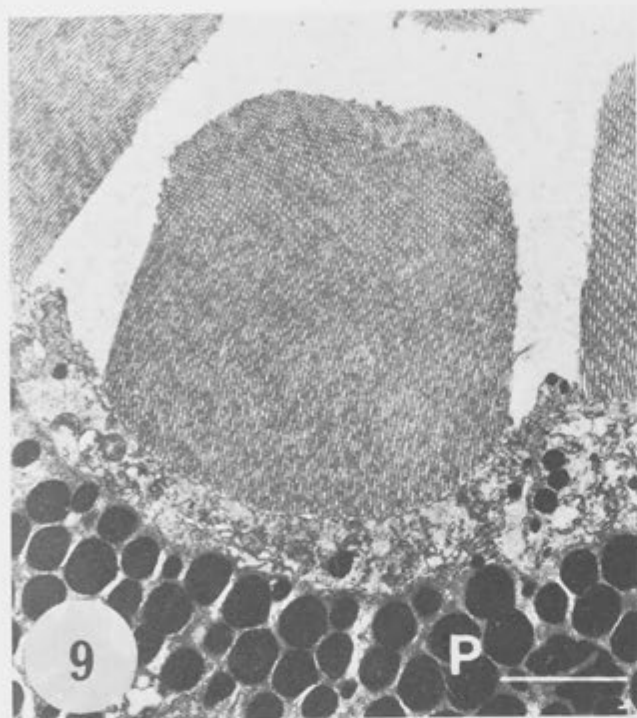


Fig. 9. TS of outer reticular cell in second rhabdom region at night. (P) pigment in surrounding pigment cells. $\times 6,600$; scale = $2 \mu\text{m}$

Fig. 10. Second rhabdom region at night in TS. The rhabdomeres are partially contiguous. (7) axon of R7. $\times 2,500$; scale = $5 \mu\text{m}$

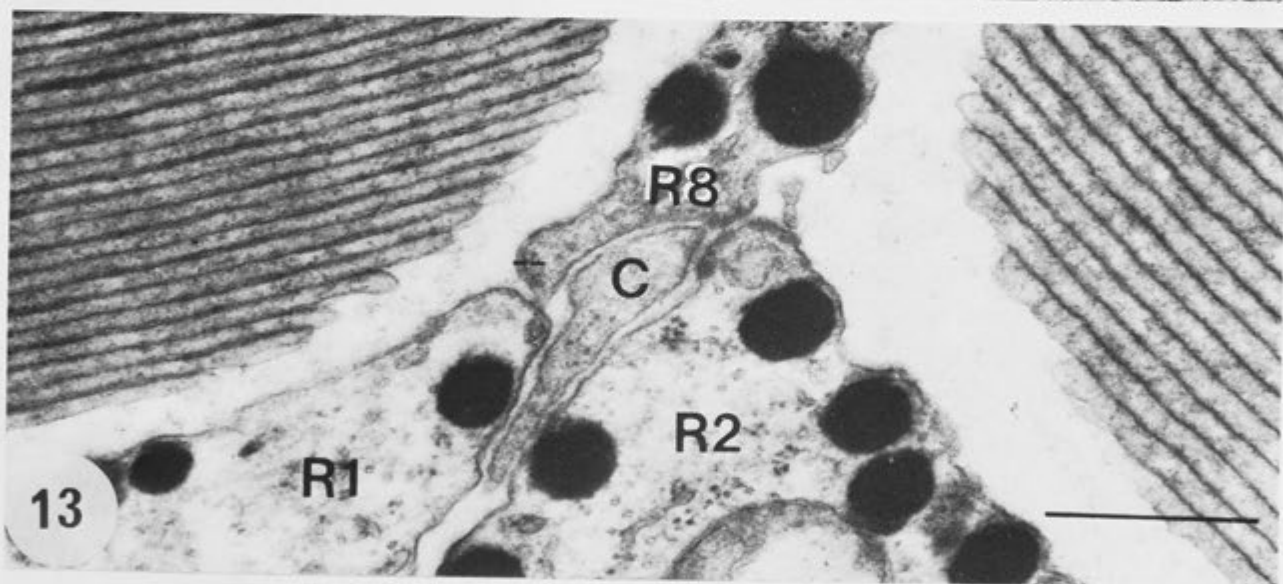
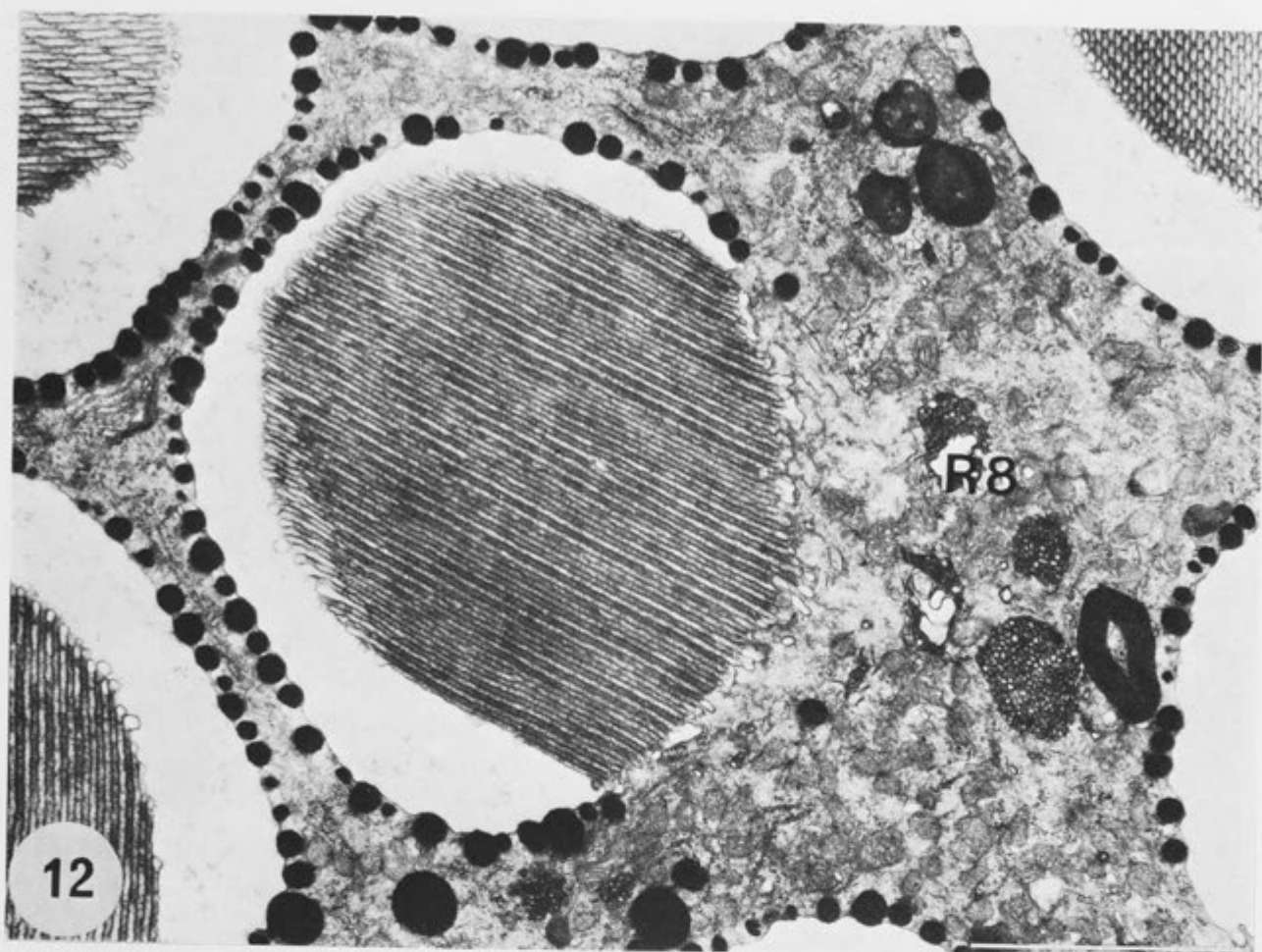


Fig. 11. Distal part of the third rhabdom region at night. Right side of section is more distal. (N_{p2}) nucleus of secondary pigment cell; ($R7$ and $R8$) seventh and eighth retinular cells. $\times 4,800$; scale = $5\ \mu\text{m}$

Fig. 12. Rhabdomere of $R8$ completely enclosed by its own cell. $\times 13,000$; scale = $2\ \mu\text{m}$ (At same level as Fig. 11, but higher magnification)

Fig. 13. Junction between processes from $R8$ and two adjacent outer retinular cells ($R1$, $R2$). Cone cell process (C) lies in the middle. $\times 41,000$; scale = $0.5\ \mu\text{m}$. (At same level as Fig. 11, but higher magnification)

Screening Effect. The overall effect of these gross cell and pigment movements is that at night the rhabdoms are optically isolated by cylindrical curtains of pigment, and during the day these curtains are raised and drawn in towards the centres of the ommatidia above the rhabdoms to minimize light reaching the rhabdom from the lenslet. Basal screening of the ommatidium, and therefore screening of antidromic light, occurs in both night and day adaptation (Sotavalta et al., 1962).

Control. Control of night- and day-adapted states is based on a combination of the immediate state of illumination and a circadian rhythm. Eyes from animals held in darkness over dawn and fixed in darkness during the day were fully light-adapted, with their ommatidial irises shut down. Eyes held under room lighting over dusk and fixed during the night, or even eyes exposed to 1 h of the same lighting, were also in the day-adapted state. Therefore, night adaptation can only be induced at night and under darkness, whereas day-adaptation requires either illumination or daytime. Light and dark adaptation have been found to be controlled similarly in the compound eyes of a scarabaeid beetle (Meyer-Rochow and Horridge, 1975), a praying mantid (Rossel, 1979), a crab (Leggett, 1978), and *Limulus* (Behrens, 1974; Barlow et al., 1977).

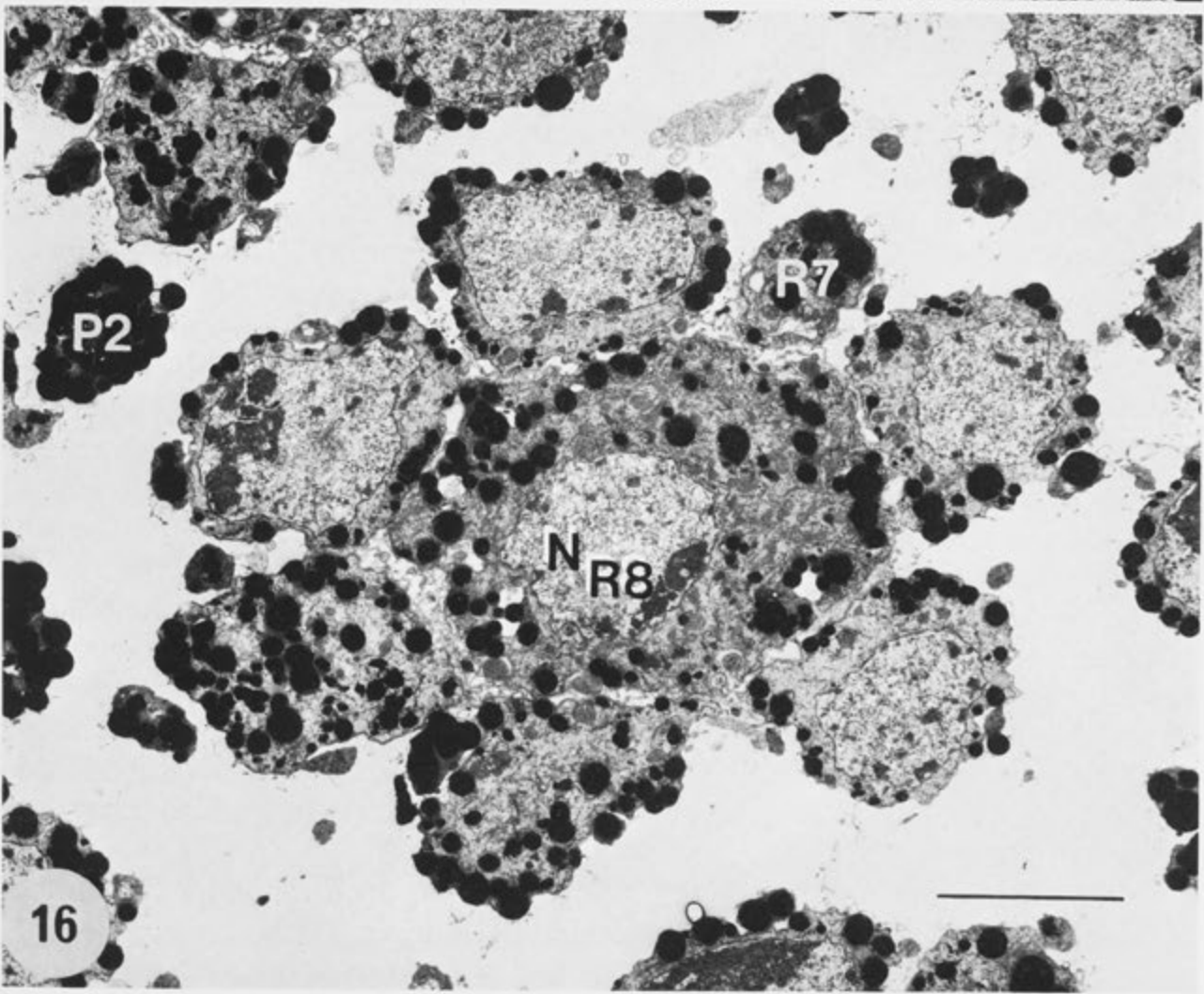
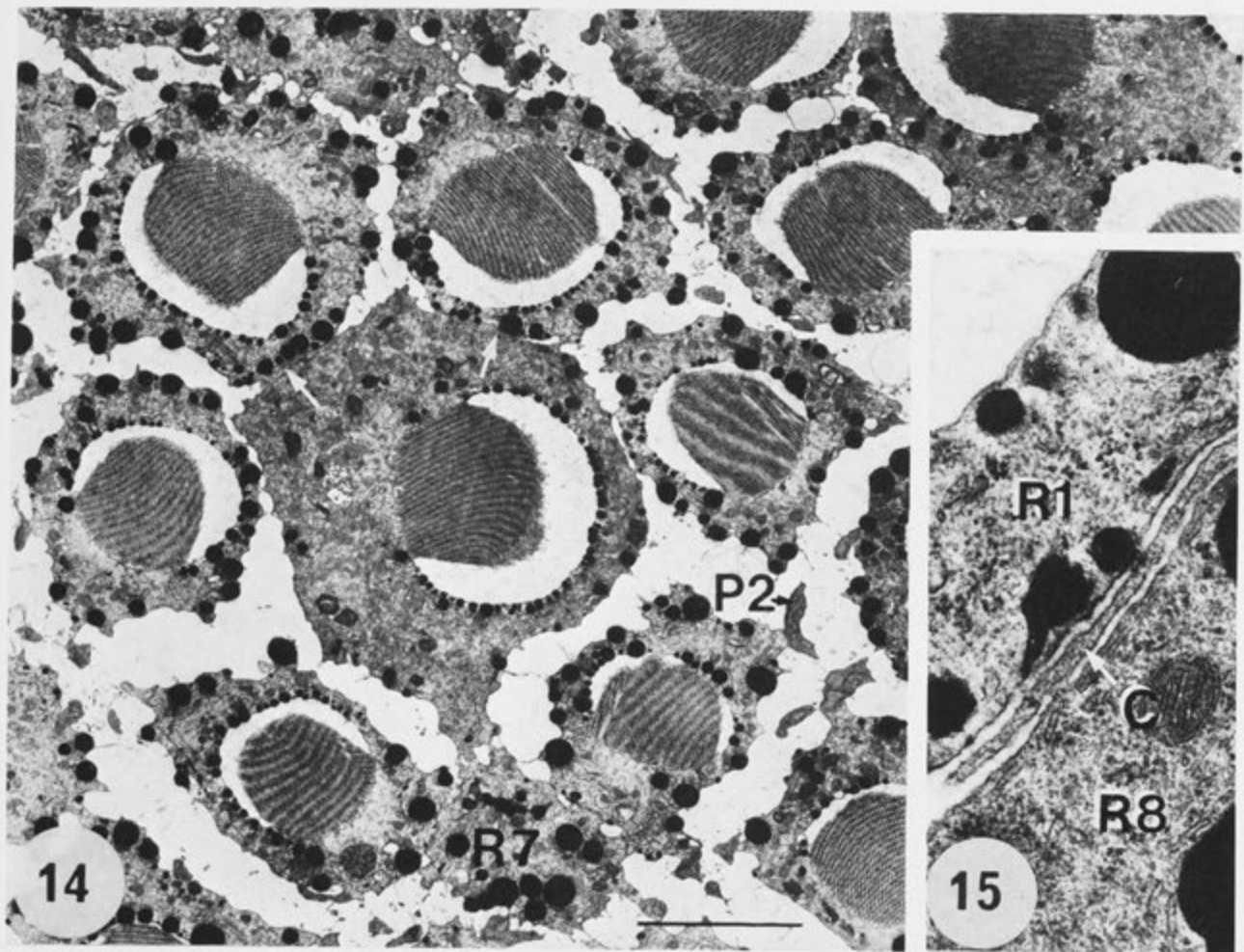
The time taken for the pigment and cell migrations to be completed is almost 1 h. Eyes on a natural light cycle were still in intermediate stages at sunrise (i.e., after 40–45 min of twilight) and 1–1.5 h after sunset.

Rhabdom Volume. At night, the rhabdomeres have significantly larger cross-sectional areas, and rhabdom length increases somewhat. Sampling six animals, 3 fixed at noon and 3 at midnight, measurements of the cross-sectional dimensions of the rhabdomeres of R1–6 were taken from a total of 100 rhabdomeres. Only rhabdomeres of undistorted ommatidia from the third rhabdom region and in precise transverse section were considered. At midnight compared to noon, microvillar length in the middle of the rhabdomere was almost 50% greater (it increased from 3.8 ± 0.05 (S.E.) μm to 5.6 ± 0.10 μm), and the rhabdomeres were just more than 25% wider (5.2 ± 0.12 μm compared to 4.1 ± 0.10 μm). Cross-sectional area therefore increased by some 87% between noon and midnight, followed by a corresponding decrease in the subsequent 12 h. Dimensions from an eye fixed at 0.400 h were slightly greater than those recorded at midnight, but not enough samples were examined to determine this with confidence. Rhabdom lengths in 2 eyes from different flies fixed at midnight were about 50 μm long, compared to 40 μm measured from rhabdoms of 2 eyes from different flies fixed at noon.

Fig. 14. TS of day-adapted retina in fourth rhabdom region. Each retinular cell encloses its own rhabdomere. Cone cell processes (arrows) terminate in this region. (P2) column of secondary pigment cell; (R7) seventh retinular cell. $\times 3,400$; scale = 5 μm

Fig. 15. Proximal end of cone cell process (C) at higher magnification than shown in Fig. 14. (R1 and R8) first and eighth retinular cells. $\times 40,000$

Fig. 16. TS of retinular cells below rhabdom in day-adapted ommatidium. Most retinular cell nuclei are apparent (N_{R8} , that of R8). Secondary pigment cells (P2) contain pigment. (R7) seventh retinular cell. $\times 3,800$; scale = 5 μm



Contents. Different organelles are predominant in the reticular cells between night and day. Most obvious during the day are massive secondary lysosomes, derived from shed photoreceptor membrane (Williams and Blest, 1980). At night, these lysosomes are absent, and there is a greater abundance of bound and free ribosomes, which are densely packed.

Pigment Granules. Neither the large nor the small pigment granules of the reticular cells appeared to move during light and dark adaptation, but it is possible that the fixation methods employed here were unable to arrest them in their different states. Pigment granules were always found lining the inside of the processes of the reticular cells that surround the rhabdomeres (Fig. 12). If these granules migrated there only under light adaptation, then they should effect a longitudinal pupil mechanism that would help to control the light flux within the rhabdomeres, as has been described in many other insects (reviewed by Stavenga, 1979).

Function. The flight activity of *Ptilogyna* peaks around dusk, beginning in daylight and extending well into the night, implying that both the day- and night-adapted eye, and their intermediate states, are functionally important.

D. Discussion

I. Comparative Aspects of Structure

Rhabdom. Sotavalta and his coworkers (1962) described only seven reticular cells in the tipulids they examined, but mistook the central rhabdomere as being derived from only one cell. The rhabdom of *Ptilogyna* can be closely compared to those described in two other nematoceran dipterans, the adult mosquito, *Aedes* (Brammer, 1970), and adult mycetophilid, *Arachnocampa* (Meyer-Rochow and Waldvogel, 1979). The main difference between the rhabdoms of *Ptilogyna* and *Arachnocampa* and that of *Aedes* is that the mosquito's is not open. The outer rhabdomeres of the mosquito rhabdom are juxtaposed to their neighbours and the central reticular cell down their whole length (Brammer, 1970), whereas in *Arachnocampa* (Meyer-Rochow and Waldvogel, 1979) and *Ptilogyna* only a short distal region of the rhabdom is thus closed. The rhabdoms of *Ptilogyna* and *Arachnocampa* are probably representative of an early stage in the evolution of the dipteran open rhabdom. The nematoceran Simuliidae and Bibionidae (Dietrich, 1909), as well as some other Tipulidae (own unpublished observations), have symmetrical rhabdoms like those of *Ptilogyna*, but which are fully open. Brachyceran flies (those in the more advanced dipteran suborder) all have fully open rhabdoms, in which a given transverse section depicts six peripheral and one central rhabdomere lying freely in ECS in a characteristic trapezoid pattern (Dietrich, 1909).

One other order, the Hemiptera, has evolved an open rhabdom containing a large central ECS, as in *Gerris* (Schneider and Langer, 1969).

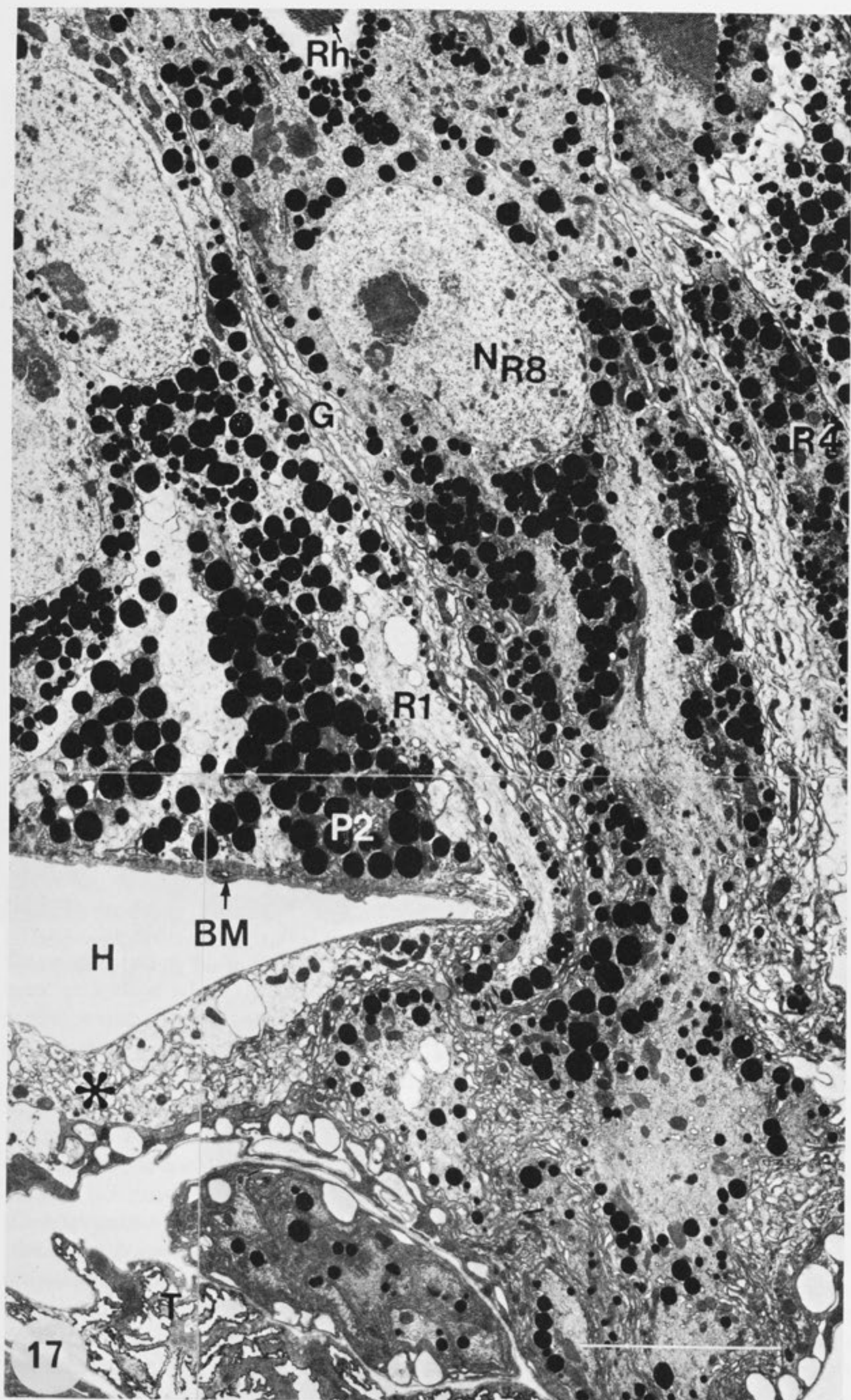


Fig. 17. Near longitudinal profile (*montage*) at base of day-adapted retina. Divided processes of glia (*G*) extend above basement membrane (*BM*) to ensheath R8 (*N_{R8}*, nucleus of R8). A continuous network of glia extends across lamina at level marked *. (*H*) haemocoel; (*P2*) secondary pigment cell; (*R1* and *R4*) first and fourth retinular cells; (*Rh*) base of rhabdomere of R8; (*T*) trachea. $\times 5,700$; scale = $5\ \mu\text{m}$

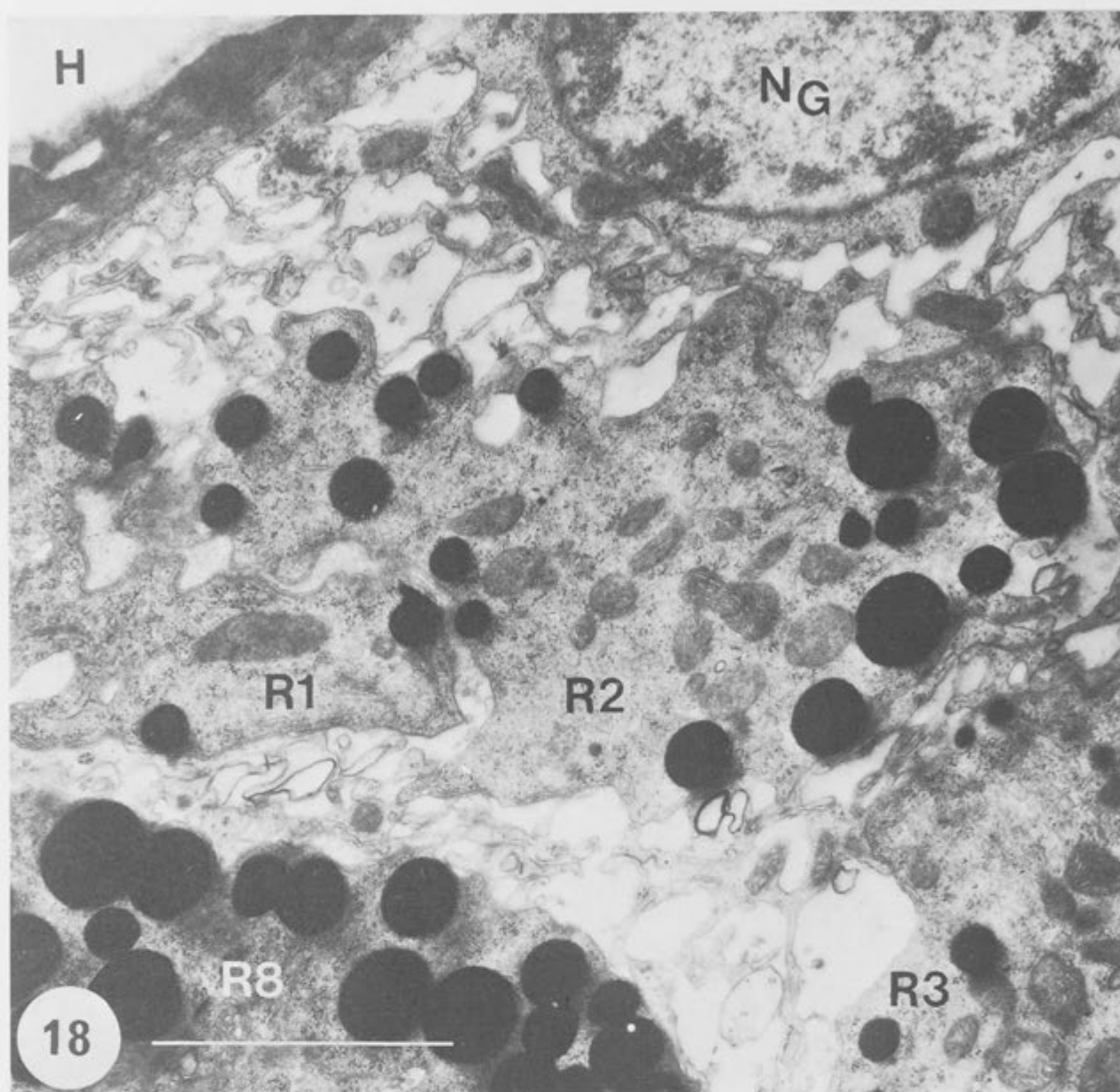


Fig. 18. Sagittal profile through the level of the lamina marked* in Fig. 17. Divided processes of glia interdigitate with receptor axons (R1, R2, R3, R8). (NG) nucleus of the glia; (H) haemocoel. $\times 17,000$; scale = $2\ \mu\text{m}$

Lamina. In muscoid flies, the first region of the lamina, or fenestrated layer, has abundant tracheae and contains glial cells, termed 'satellite cells', that ensheath the neural elements between the fenestrated layer and the next lamina region, the layer of cell somata (Trujillo-Cenóz, 1965; Boschek, 1971). The first region of the lamina in *Ptilogyna* clearly differs by having (1) a glial network that extends through the basement membrane into the retina and (2) limited tracheation, but large spaces that are possibly haemolymph channels.

By location, the glia described in the first region of the lamina in *Ptilogyna* may be considered to correspond to the satellite glial cells. But, Trujillo-Cenóz (1965) describes these cells as 'poor in mitochondria and particulate elements'. On the other hand, epithelial glial cells in the plexiform layer (optic cartridge region) of muscoids are particularly rich in particulate elements (which he suggests are ribosomes) and mitochondria, and send out processes (capitate projec-

tions) into invaginations in the photoreceptor axons (Trujillo-Cenóz, 1965). These epithelial cells are therefore perhaps more closely related functionally to the glia described here in *Ptilogyna*, which also protrudes into invaginations of the photoreceptors and contains abundant mitochondria and ribosomes.

II. Effects of Night and Day Adaptation

Cell and Pigment Migrations. Large distal-proximal migrations of the rhabdom and iris-like behaviour of the PPCs during night-day adaptation have also been described in the hemipteran *Lethocerus* (Walcott, 1971a), and are known to cause the angular acceptance of the retinular cells to change greatly between light (3.5°) and dark (9.0°) adaptation (Walcott, 1971b). An analogous change should also occur in *Ptilogyna*. Ioannides and Horridge (1975) found the distal part of a dark-adapted rhabdom of *Lethocerus* to be well inside the focal plane of its lenslet. The distal end of a night-adapted rhabdom in *Ptilogyna*, which is only 3–4 μm from the base of the lenslet, was shown to be in a similar out-of-focus position when the lenslets of an eye were hung from a drop of saline beneath a microscope objective (cf., Homann, 1928). In-focus images of a distant object were observed at a real distance of 30–35 μm behind each lenslet. In this out-of-focus position, rhabdomeres should have larger receptive fields than if their ends were in the plane of focus. In the light-adapted ommatidia of *Ptilogyna*, the acceptance angles of retinular cells should be drastically decreased by the screening pigment in the PPCs. (Note that the PPCs differ from the vertebrate iris in that they form a 'field stop' rather than an 'aperture stop'.)

Altering the acceptance angles of the retinular cells according to different light intensities occurs in many animals (Walcott, 1975). The advantage of these changes in view of a "trade-off" with resolution has been discussed by Snyder (1979). In short, increasing the acceptance angles at night increases the proportion of photons from an extended field impinging upon each rhabdomere at a time when the field's luminance decreases. A decrease in luminance causes a decrease in signal to noise ratio, but the increase in photon capture efficiency helps to improve this ratio.

Changes in Rhabdom Volume. A reduction in rhabdom size upon exposure to light and an increase during darkness has been reported in the larval mosquito ocellus (White and Lord, 1975), the adult mosquito compound eye (Brammer et al., 1978), and the compound eye of the mycetophilid, *Arachnocampa* (Meyer-Rochow and Waldvogel, 1979). Furthermore, changes in rhabdom volume occur between night and day in the spider, *Dinopis* (Blest, 1978), and the crabs, *Grapsus* (Nässel and Waterman, 1979) and *Leptograpsus* (Blest et al., 1980; Stowe, 1980), when maintained on a normal daily light cycle. Both *Dinopis* and the two crabs are active at night, and Blest (1980) proposed that variable-volume rhabdoms may be designed for use at night or over a wide range of light intensities. The finding here that there exist differences in the gross dimensions of the rhabdom of *Ptilogyna* between midday and midnight supports this viewpoint.

and provides the first example of rhabdom volume changes in an insect on normal daily light cycle. More recently, such changes have also been found in the locusts, *Valanga* (G.A. Horridge, L. Marcelja, J. Duniec, unpublished) and *Locusta* (own observations), which fly at night when conditions of temperature and humidity are suitable (Davey, 1959; Clark, 1969).

Fluctuations in rhabdom volume occur as a result of asynchrony of the shedding and synthetic phases of photoreceptor membrane turnover. In *Ptilogyna*, microvilli are short at midday because most photoreceptor membrane is shed each morning by the detachment of their distal segments (Williams and Blest, 1980). Apparently, the length of the microvilli is restored considerably during synthesis prior to midnight, although there was some evidence to suggest that it continues to increase until 0.400 h. The changes observed in rhabdomeral width (perpendicular to the microvillar long axis) and length of the rhabdom suggest that in a daily cycle whole new microvilli are added and detached. In *Grapsus*, the number of microvilli doubles at night (Nässel and Waterman, 1979).

In *Dinopis*, Blest (1978) considered any increase in absolute sensitivity as a result of the increased photon capturing efficiency of a larger rhabdom at night to be small in relation to the shift in sensitivity range likely to be attained by alterations in membrane performance alone during dark adaptation. The same is probably true for *Ptilogyna*. An eye such as *Ptilogyna*'s that appears to remain functional after dusk should be capable of shifting its sensitivity range through several log. units between day and night. The increased photon capture from an increase in rhabdomeral cross-sectional area of 87% and rhabdom length from 40 to 50 μm that occurs in *Ptilogyna* at night would provide only a minor proportion of a shift in sensitivity range of this magnitude. However, the increased photon-capture efficiency of the larger rhabdom may be more important by helping improve signal to noise ratio at night, thus enhancing the increase in this ratio brought about by changes in reticular cell angular acceptance resulting from cell migrations (see above). The increase in rhabdomeral cross-sectional area during rhabdom growth would, of course, manifest itself by further increasing the acceptance angles of reticular cells.

Conclusion. Movement of reticular cell screening pigment (Kirschfeld and Franceschini, 1969), is the only structural change to have been reported in advanced dipterans during light and dark adaptation. These pigment movements appear to produce an increase in the acceptance angle of reticular cells during dark adaptation, but it is rather modest; about 25% in *Calliphora* (Hardie, 1979). In this paper extensive movements of the ommatidial cells and changes in rhabdom size were shown to occur between night and day in a more primitive dipteran. These changes should dramatically affect visual performance in *Ptilogyna*, in a manner consistent with this fly's activity at dusk, which extends over a wide range of environmental light intensities.

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CHAPTER III

Twisted Rhabdomeres in the Compound Eye of a Tipulid Fly (Diptera)

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Summary. The individual rhabdomeres of the outer retinular cells (R 1–6) in the tipulid fly, *Ptilogyna*, twist about their long axes. Proximally, the rhabdoms become partitioned off by processes from the retinular cells, so that the basal region of each rhabdomere is enclosed in a “pocket” formed by its own cell (Fig. 2). This organisation of the rhabdom enables each rhabdomere to twist while supported within its own retinular cell, and while the cell itself maintains its orientation with respect to the entire ommatidium. Theory predicts that the rhabdomeral twisting should significantly reduce the polarisation sensitivity of R 1–6, but have little effect on the efficiency with which unpolarised light is absorbed.

Key words: Photoreceptors – Rhabdomeral twisting – Compound eye – Tipulid fly (Diptera) – Polarisation sensitivity

Twisted rhabdoms have been described from electron microscopy in the compound eyes of the worker bee (Grundler 1974; Wehner et al. 1975; Wehner 1976), bull ant (Menzel 1975; Menzel and Blakers 1975), and damselfly (Ninomiya et al. 1969). In addition, it has been claimed that the individual rhabdomeres of *Calliphora* and *Drosophila* twist (Smola 1977; Smola and Tscharrntke 1979); however, the orientation of rhabdomeres in the open rhabdoms of advanced dipterans is susceptible to distortion during conventional histological preparation (Boschek in McIntyre and Snyder 1978; own observations). Recently, Ribi (1979) refuted the reality of twisting of rhabdomeric structures in both *Calliphora* and the worker bee, suggesting that the twist observed by other workers was artefactual.

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The present paper reports that rhabdomeres twist in the open rhabdom of the tipulid fly, *Ptilogyna*, and shows that their twisting is based on an unusual organisation of the retinula.

Materials and Methods

Adult *Ptilogyna gracilis spectabilis* (Skuse) (Dobroworski 1971) were collected from the banks of the Mongarlowe River, near Braidwood, New South Wales. Their compound eyes were dissected in cold primary fixative. Care was taken not to distort the eyes during dissections. Usually, a small amount of head capsule was left intact around the margin of the eye and the optic lobes were not cut away. In some flies a few small holes were made in the cornea to increase penetration of the fixative. Others were only decapitated. Although the quality of retinal fixation of the latter was poor because of slow fixative penetration, the eyes could be expected to be free of any mechanical distortion incurred from dissection of the head. The primary fixative was 2.5% glutaraldehyde + 2% paraformaldehyde buffered in 0.1 M sodium dihydrogen orthophosphate-NaOH with 0.07 M D-glucose and 0.9 mM CaCl_2 at pH 7.3. Primary fixation time was restricted to 2 h in order to prevent artefacts resulting from delaying osmication (Williams 1980a). After a brief wash in buffer, tissues were postfixed for 1–2 h in 1% OsO_4 in the same buffer. They were embedded in Araldite, and silver sections were cut with glass knives and collected on formvar-coated slot grids. Both serial transverse sections from the central region of the eye and longitudinal sections were examined in a Jeol 100C or Hitachi H500 electron microscope.

Results

The structure of the rhabdom of *Ptilogyna* has been described in detail previously (Williams 1980b). Essentially, it accords with the basic dipteran arrangement, consisting of six outer rhabdomeres from retinular cells (= photoreceptor cells) 1–6 (R1–6) and a central tiered rhabdomere from R 7 distally, and R 8 proximally. It is 40–50 μm long and may be divided into four regions (Williams 1980b). In the most distal 8 μm , the rhabdomeres of R1–6 and R 7 form a closed rhabdom (region 1). Below this region, the rhabdomere of R 8 replaces that of R 7. The rhabdomeres of R1–6 and R 8 are not contiguous. For about 7 μm (region 2) the rhabdomeres lie freely in a large common extracellular space (ECS). Proximal to region 2, this ECS is partitioned off by retinular cell processes. In region 3, processes from R 8 extend around its own rhabdomere and to the perimeter of the rhabdom between the rhabdomeres of R1–6. Then, in a transitional zone with an upper limit about 20 μm from the distal end of the rhabdom, the lateral processes of R 8 are replaced by processes from R1–6 (Fig. 1), so that in region 4 each retinular cell completely encloses its own rhabdomere (Fig. 2).

In region 1, the microvilli of R1–6 are all directed towards the centre of the rhabdom (Williams 1980b); however, in the partitioned regions 3 and 4, deviation from this orientation is obvious as the rhabdomeres twist about their long axes (Figs. 1, 2, 3). The tiered rhabdomeres of R 7 and 8, which are perpendicular to each other, do not twist. The microvilli of R 7 maintain an orientation perpendicular to the equator of the eye, which is divided into dorsal and ventral halves (cf. Dietrich 1909).

The patterns of twisting of the six outer rhabdomeres were examined in transverse sections of different ommatidia in the central region of the eye and are illustrated in Fig. 4. The rhabdomeres may be considered in pairs: those from R 1 and 6, R 2 and 5 and R 3 and 4. Rhabdomeres of each pair twist in opposite

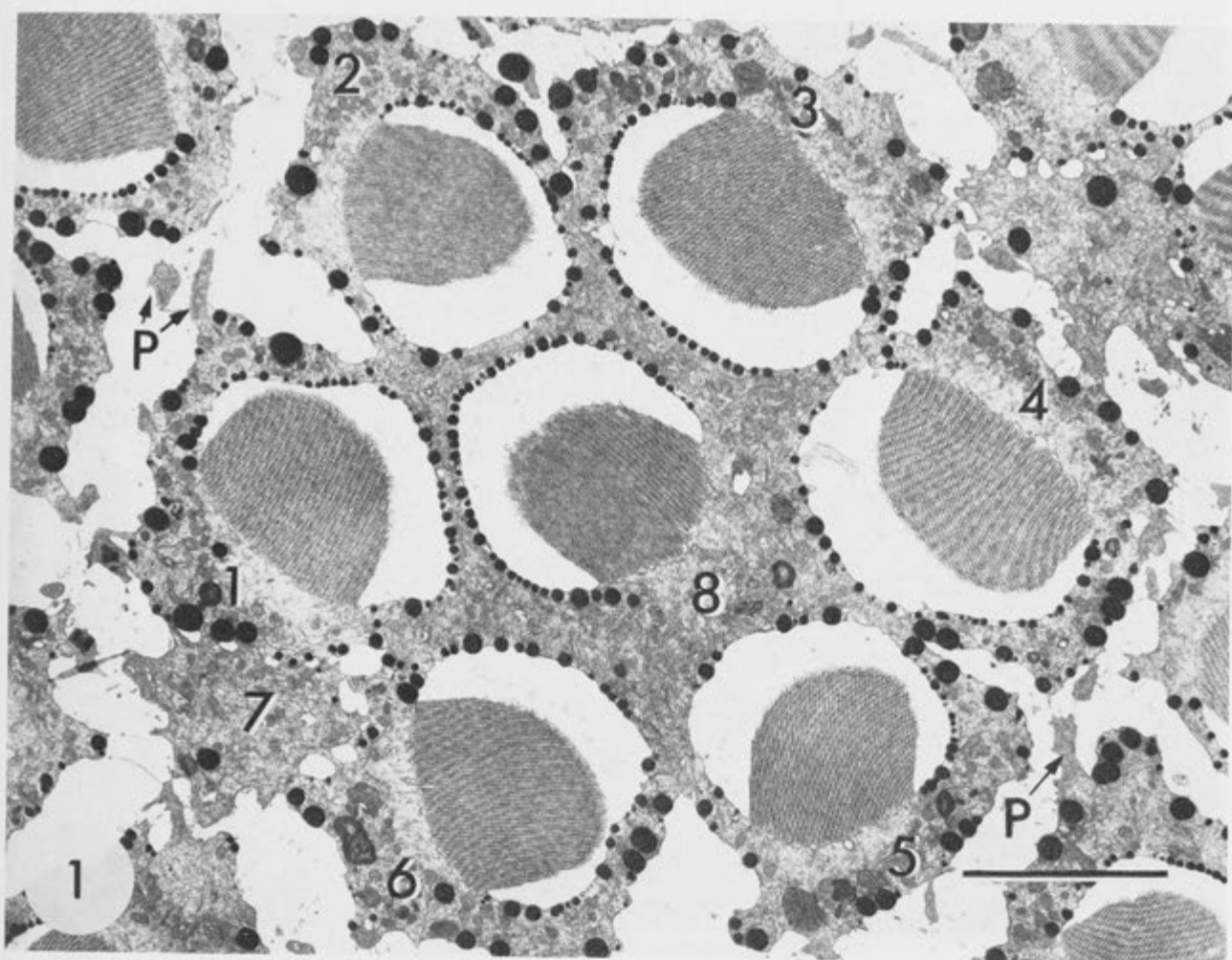


Fig. 1. Electron micrograph of a transverse section cut midway through the rhabdoms of *Ptilogyna* fixed during the day. Retinular cells of a single ommatidium are numbered. *R*7 is limited to its axon and *R*8 provides the central rhabdomere. Characteristic of day-adapted retinae, the contents of the secondary pigment cells are withdrawn distally and concentrated between the lenslet and rhabdom (Williams 1980b), so that only narrow non-pigmented columns of pigment cells (*P*) and extracellular space remain between neighbouring rhabdoms. The retinular cell extensions around each outer rhabdomere are of unequal length, with the result that the rhabdomeres are oriented away from the centre of the ommatidium. $\times 4,200$; scale = $5\mu\text{m}$.

directions, such that one is the mirror image of the other about the plane that bisects the rhabdom through *R*7. Deviation from this symmetrical pattern, such as illustrated by the rhabdomeres of *R*2 and 5 in ommatidium B in Fig. 2b, is only very rare. The following general conclusions were made: (1) Rhabdomeres from *R*1 and 6, and *R*3 and 4, can twist either way. (2) The direction of twist of the rhabdomeres from *R*2 and 5 is usually the same: their bases are shifted towards the side of the ommatidium that bears the axon of *R*7. Therefore, there are four possible major ommatidial patterns of rhabdomeral twisting, the permutations resulting from the different twist directions of the rhabdomeres of *R*1 and 6 and *R*3 and 4. However, because the rhabdomeres of *R*3 and 4 twist their bases more frequently away from *R*2 and 5 (76% of 300 ommatidia noted), two combinations of the patterns are more common than the other two. No attempt was made to discover if there was any relationship between particular patterns of twist and given regions of the eye.

At the proximal end of the rhabdom, the orientation of the outer rhabdomeres differs by some $100\text{--}120^\circ$ from that in the distal rhabdom. In ommatidium D in Fig. 2b, which is shown about $10\mu\text{m}$ from the proximal end of the rhabdom, the

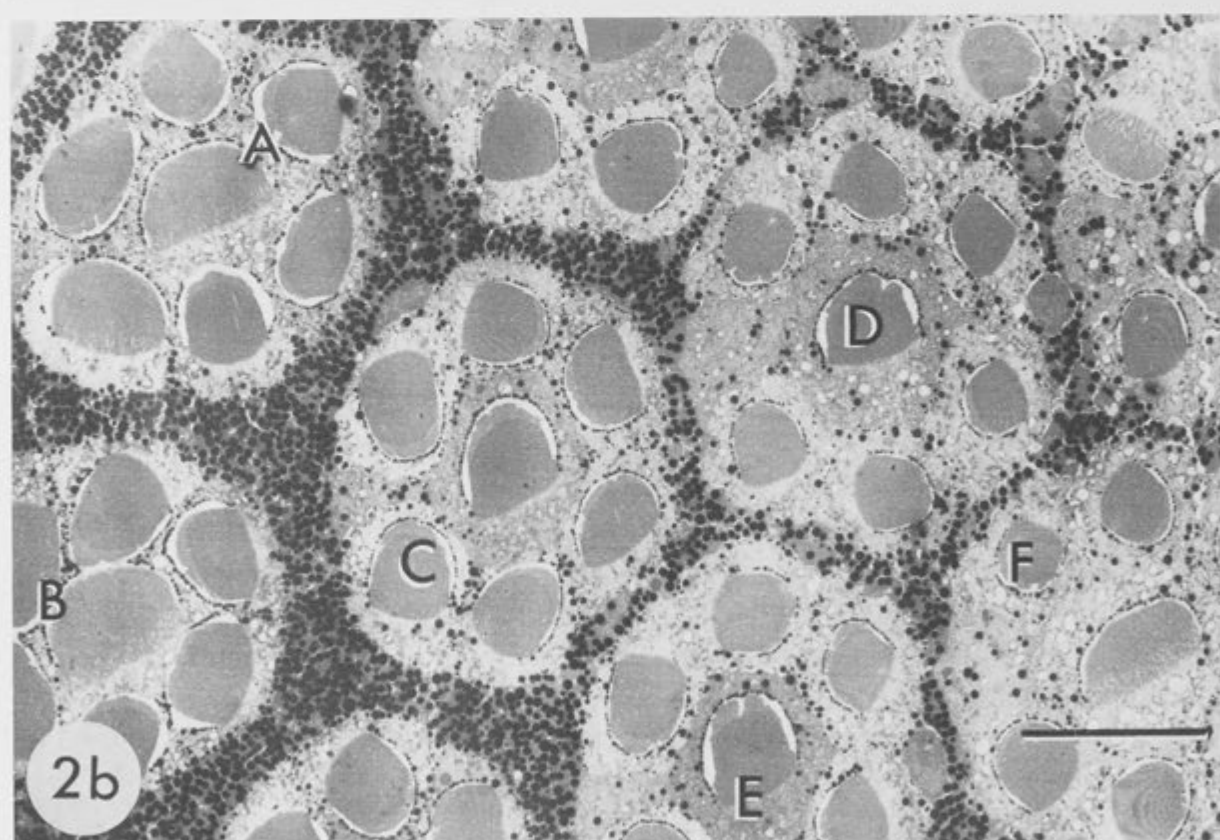
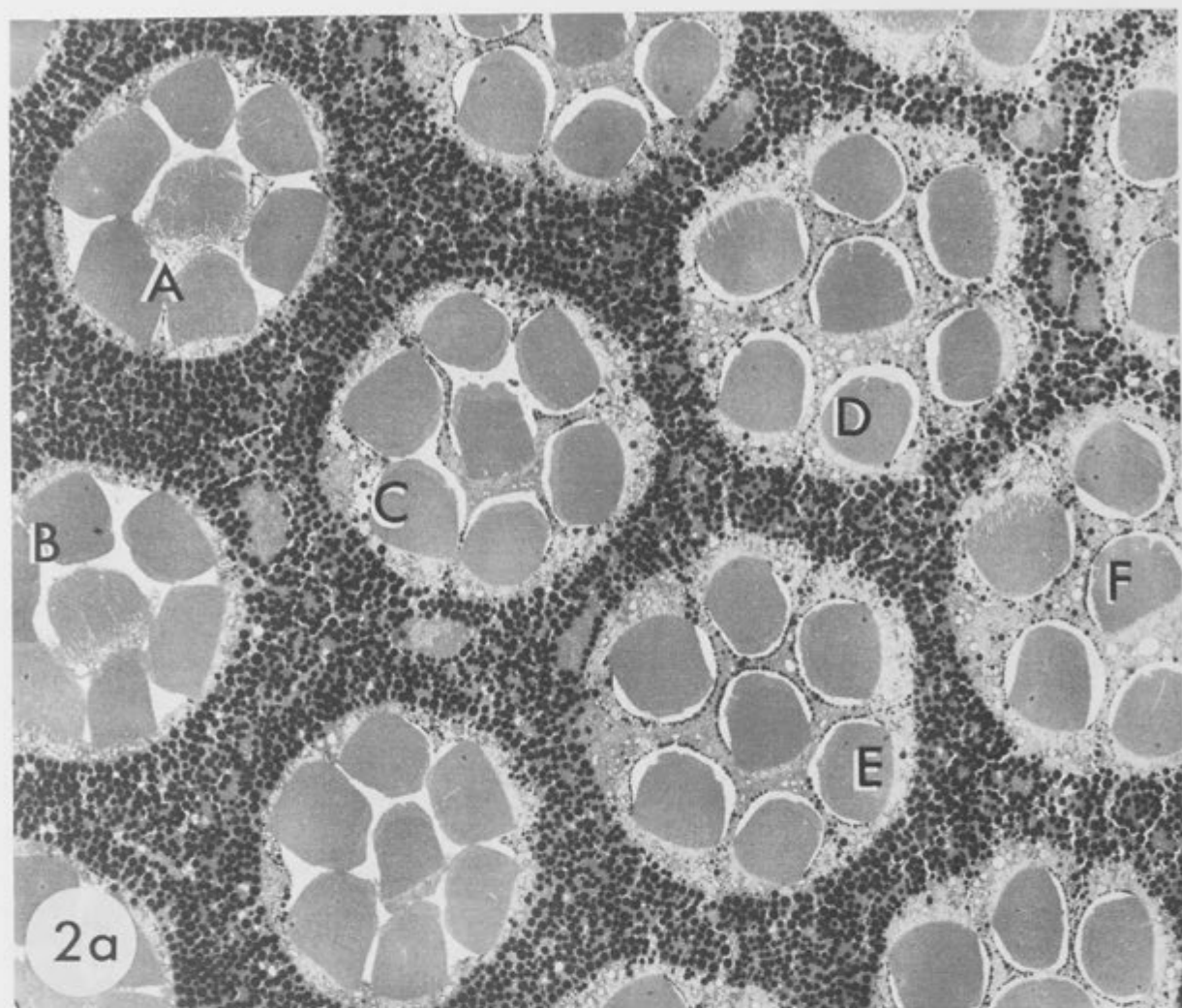


Fig. 2. Electron micrographs of transverse sections through a night-adapted retina of *Ptilogyne*. $\times 1,800$; scale = $10\text{ }\mu\text{m}$. **a** The centre of the section is in the top right corner, so that of the ommatidia marked (A–F), D is the nearest to being in true transverse section; the others are cut rather obliquely due to the large interommatidial angles. Ommatidia A and B are shown through region 2, C between regions 2 and 3, and D, E, and F through region 3. **b** Same region of the retina as in **a** but sectioned about $7\text{ }\mu\text{m}$ more proximally. Ommatidia A and B are shown through region 3, and C, D, E, and F through region 4

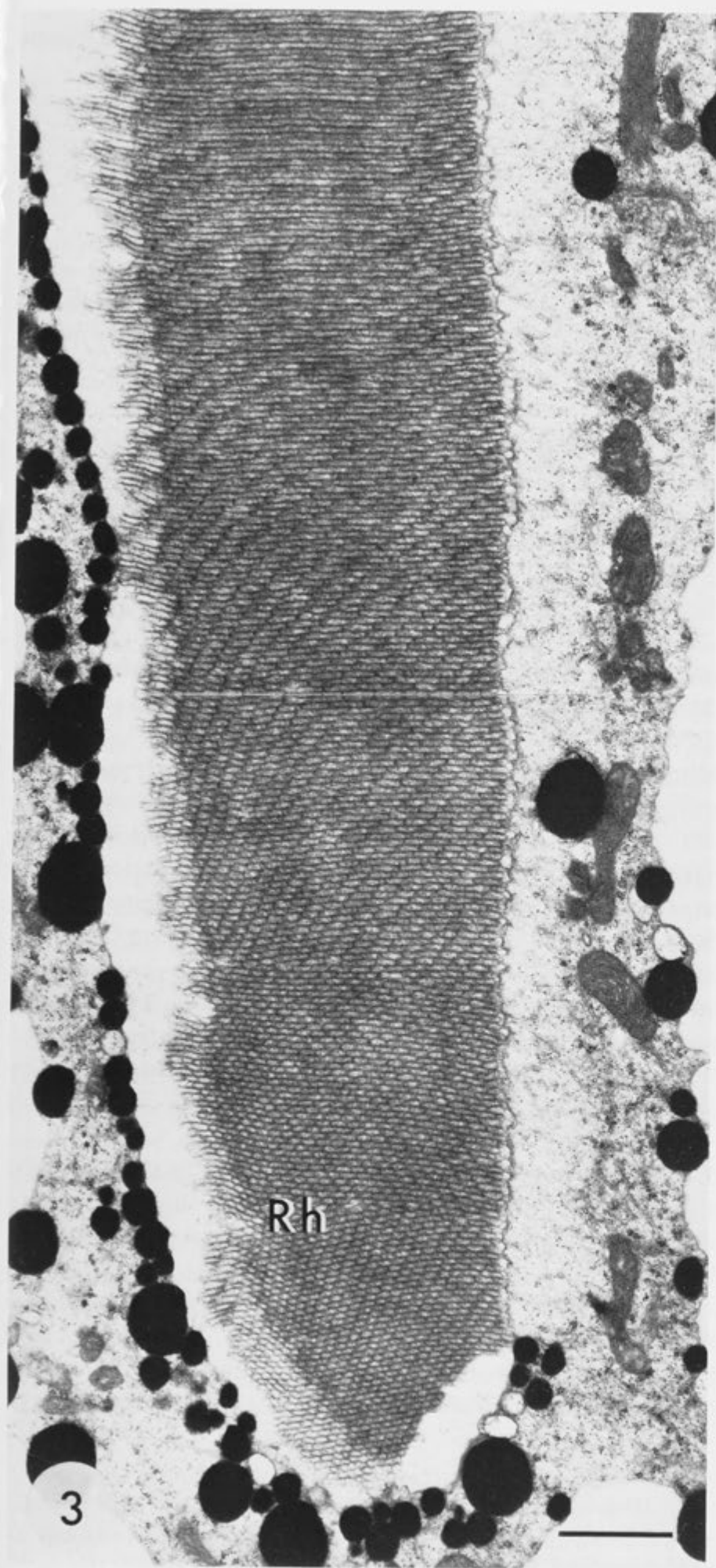


Fig. 3. Electron micrograph of a longitudinal section of an outer (R1-6) reticular cell of *Ptilogyna* showing the proximal end of its rhabdomere (*Rh*). Distally (upper) the microvilli are parallel to the plane of section, proximally (lower) they are nearly perpendicular to it, indicating a rhabdomeral twist of almost 90° down the length shown. The distal ends of the microvilli appear disordered and pale as they are about to be shed from the rhabdom (Williams and Blest 1980). $\times 14,000$; scale = $1\ \mu\text{m}$

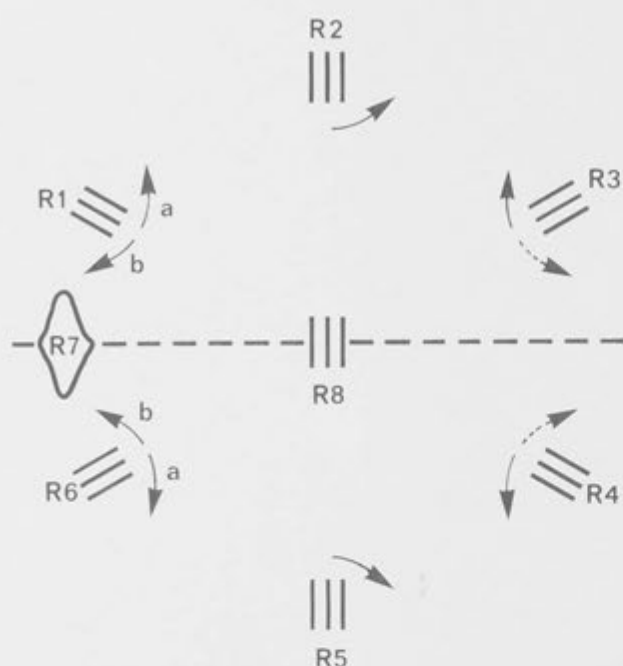


Fig. 4. Diagram illustrating the common patterns of rhabdomeral twisting observed in different ommatidia in the centre of the eye of *Ptilogyna*. Arrows indicate directions of twist; directions indicated by broken arrows were less common. Rhabdomeres within each pair, R1 and 6, R2 and 5, R3 and 4, twist in opposite directions. Thus, R1-6 maintain mirror symmetry about the dashed line that corresponds to a plane bisecting the rhabdom through R7. The tiered rhabdomeres of R7 and 8 do not twist, and their microvilli are always orthogonal.

microvilli of R1-6 are oriented nearly perpendicularly to radii from the centre of the ommatidium. The rate of twist averages more than $2^\circ \mu\text{m}^{-1}$ for the entire rhabdom, but it increases progressively towards the proximal end of the rhabdom.

The observed twisting is unlikely to be artefactual for two reasons: (1) Rhabdomeres of R1-6, even from different specimens, were always observed to be twisted by about the same amount ($100-120^\circ$), whereas the central rhabdomere was never twisted. If the rhabdomeres appeared twisted because of distortion during histological preparation, one would not expect to find such consistency among different samples. (2) More importantly, the reticular cells did not appear distorted. They were held together by numerous junctions, often including desmosomes, between adjacent reticular cells and cone cell processes (Williams 1980b). The positions of R1-6 appeared securely fixed with respect to the central cell, R8 (Fig. 1). In night-adapted samples, the whole rhabdom is supported further by secondary pigment cells, which are packed tightly around it (Fig. 2) (Williams 1980b).

Rhabdomeral twisting in *Ptilogyna* is based on a framework provided by the reticular cell processes that divide the rhabdom. Fig. 1 is taken slightly distal to region 4. At this level the incomplete processes from each of R1-6 are of unequal length, and consequently cause the orientation of their rhabdomeres to differ from that in the distal rhabdom. Thus, each rhabdomere of R1-6 effectively twists *within* its own cell, while the cell maintains cohesion with the remainder of the ommatidium. It is noteworthy that in some other tipulids, in which the central ommatidial ECS is not divided, the rhabdomeres do not twist (own observations).

Discussion

In view of the controversy surrounding the reality of rhabdomeral twisting as reported by others (see Wehner and Bernard 1980), this report provides the most conclusive evidence to date that it is possible for microvilli in a rhabdom to be twisted and therefore not necessarily conform to a lattice in which all microvilli

from a given retinular cell must be parallel. This property is also manifest, to a lesser extent, in the closed rhabdoms of the locusts, *Locusta* and *Valanga*. Distally, during the day, these rhabdoms each comprise six rhabdomeres forming a triradiate pattern with adjacent pairs of rhabdomeres sharing the same microvillar orientation. In the proximal half, microvillar orientation of a rhabdomere may differ by some 60° to that of *both* its neighbours, and the triradiate pattern is lost (Wilson et al. 1978; own observations).

Provided that the twist rate is sufficiently large and the rhabdomeric structure has a low overall birefringence and high dichroic ratio, an effect of twisting the rhabdomeres is to reduce the polarisation sensitivity (PS) of the retinular cells. As an example, the following values can be assumed for the rhabdomeres of R1–6 in *Ptilogyna*: (1) an overall birefringence (intrinsic plus form), Δn , of 4×10^{-3} (Kirschfeld and Snyder (1975) measured a value of 4×10^{-3} for rhabdomeres of R1–6 in muscoid flies); (2) an absorption coefficient for linearly polarised light with its *E*-vector parallel to the microvillar axis, α_{\parallel} , of $0.0075 \mu\text{m}^{-1}$ (Kirschfeld (1969) gives $0.0075 \mu\text{m}^{-1}$ for muscoid rhabdomeres); and (3) a dichroic ratio of 10 (the maximum dichroic ratio possible is 20 (Snyder and Laughlin 1975)). The theory of McIntyre and Snyder (1978) predicts that such rhabdomeres, $50 \mu\text{m}$ long, should have a PS of 8.5 if not twisted, whereas when twisted by 120° at a constant rate their PS is only 1.3. In *Ptilogyna*, most of the twisting occurs in the proximal rhabdom where less light is absorbed, so that the PS reduction should not be quite this large. Moreover, if the dichroic ratio of its rhabdomeric membrane is more or less than 10, the reduction in PS is more or less accordingly.

A second effect of rhabdomeral twisting is to increase the total absorption of unpolarized light (Snyder and Laughlin 1975). Comparisons of night- and day-adapted ommatidia imply that this effect might be exploited by *Ptilogyna*. The fly is active throughout a wide range of light intensities, but only at night does light appear not to be excluded from the outer rhabdomeres by pigment cells that form a very narrow field stop (Williams 1980b). Thus, R1–6 seem to be reserved for use in scotopic vision when maximum light absorption is of prime importance. Nevertheless, if the values for Δn and α_{\parallel} given above and a dichroic ratio of 10 are assumed, the theoretical treatment of McIntyre and Snyder (1978) predicts that a 120° twist by an R1–6 rhabdomere of *Ptilogyna* should effect only a 4% increase in total light absorption over the estimate for a similar rhabdomere without twist.

In conclusion, the most likely effect of twisting the rhabdomeres of R1–6 in *Ptilogyna* is to decrease their PS.

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Ca^{++} -induced Structural Changes in Photoreceptor Microvilli of Diptera

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Summary. When the compound eyes of the fly *Lucilia* are fixed for electron microscopy with glutaraldehyde in common buffer solutions, artefactual whorls are liable to be formed from the photoreceptor microvilli. The whorls result from two factors: (i) a prolonged time interval prior to osmication, such as the “overnight” primary fixation or wash at 4°C commonly used in studies of compound eyes; (ii) as little as 1–2 mM Ca^{++} in the primary fixative and wash solutions. Osmication after short (1 h) glutaraldehyde fixation at 4°C, or omission of Ca^{++} and addition of 2 mM EGTA, prevent whorl-formation. In the tipulid fly *Ptilogyna*, similar artefacts are produced, but are confined to the distal zone of the microvilli that sheds during turnover.

Key words: Compound eye – Photoreceptor membrane – Electron microscopy – Calcium-induced changes – Artefacts – Diptera.

Since its introduction by Sabatini et al. (1963), primary fixation in buffered glutaraldehyde prior to osmication has become a standard procedure for biological electron microscopy. Nevertheless, despite the advantages of this method, there have been several indications of undesirable effects from its use (cf. Trump and Ericsson, 1965; Trump and Bulger, 1966; Bodian, 1970). They have not been explicitly noted in the literature on arthropod compound eyes, the ultrastructure of which is now frequently described from material fixed in modified Karnovsky's (1965) solution containing calcium ions, often overnight for convenience and to ensure good penetration of whole retinæ. Studies on transductive membrane synthesis and shedding (e.g., Williams and Blest, 1980) necessitate a critical examination of possible sources of artefacts. The present communication describes

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artefactual transformations of photoreceptor microvilli in two species of fly following primary fixation with glutaraldehyde in standard buffers.

Materials and Methods

Animals. Compound eyes from both sexes of wild and laboratory-bred *Lucilia sericata* (Meig.) were examined, as well as eyes of a second dipteran, the tipulid *Ptilogyna spectabilis* (Skuse). The tipulids were collected from the banks of the Mongarlowe River, near Braidwood, New South Wales.

Immersed in fixative at 4°C and pH 7.3, the eyes were rapidly dissected from unanaesthetised animals at about midday and under light filtered by a Schott KG 3 filter (absorbs heat). They were then cut into small pieces.

Fixation. Primary fixative were:

1. 2.5% glutaraldehyde buffered in 0.1 M sodium cacodylate buffer with 0.14 M sucrose and 2 mM CaCl_2 (cf. Karnovsky, 1965).

2. 2.5% glutaraldehyde and 2% formaldehyde (Karnovsky, 1965) buffered in 0.1 M sodium dihydrogen orthophosphate-NaOH with 0.07 M D-glucose and 0.9 mM CaCl_2 (Millonig, 1962).

3. 2.5% glutaraldehyde and 0.8% OsO_4 mixed immediately prior to use and buffered in 0.1 M sodium cacodylate with 0.14 M sucrose and 2 mM CaCl_2 (modified from Franke et al., 1969).

After primary fixation, tissues were washed in the same buffer in which they were fixed, and postfixed for 1 h in 1% OsO_4 , also in the same buffer.

The effects of different time intervals prior to osmication were tested by comparing the following primary fixation and washing procedures:

- (a) no primary fixation (osmication only);
- (b) fixation for 1 h in fixative 3, then brief washing;
- (c) fixation for 1 h, 7 h or 20 h, using fixative 1 and 2, then brief washing;
- (d) fixation for 1 h, using fixatives 1 and 2, then washing for 1–2 mins, 6 h or 19 h.

In a second experiments, the effects of different buffer concentrations of calcium ions were tested by varying the CaCl_2 content of primary fixative 1. It contained 0, 0.5, 2 (as above), 4 or 10 mM CaCl_2 , or 2 mM EGTA. Fixation was for 20 h at 4°C before a brief wash in buffer and osmication. Some samples fixed without CaCl_2 or with 2 mM EGTA were left in fixative for 3 days before osmication. Both experiments were carried out in full with *Lucilia* only, and comparisons were conducted between pieces of retina from the same animal.

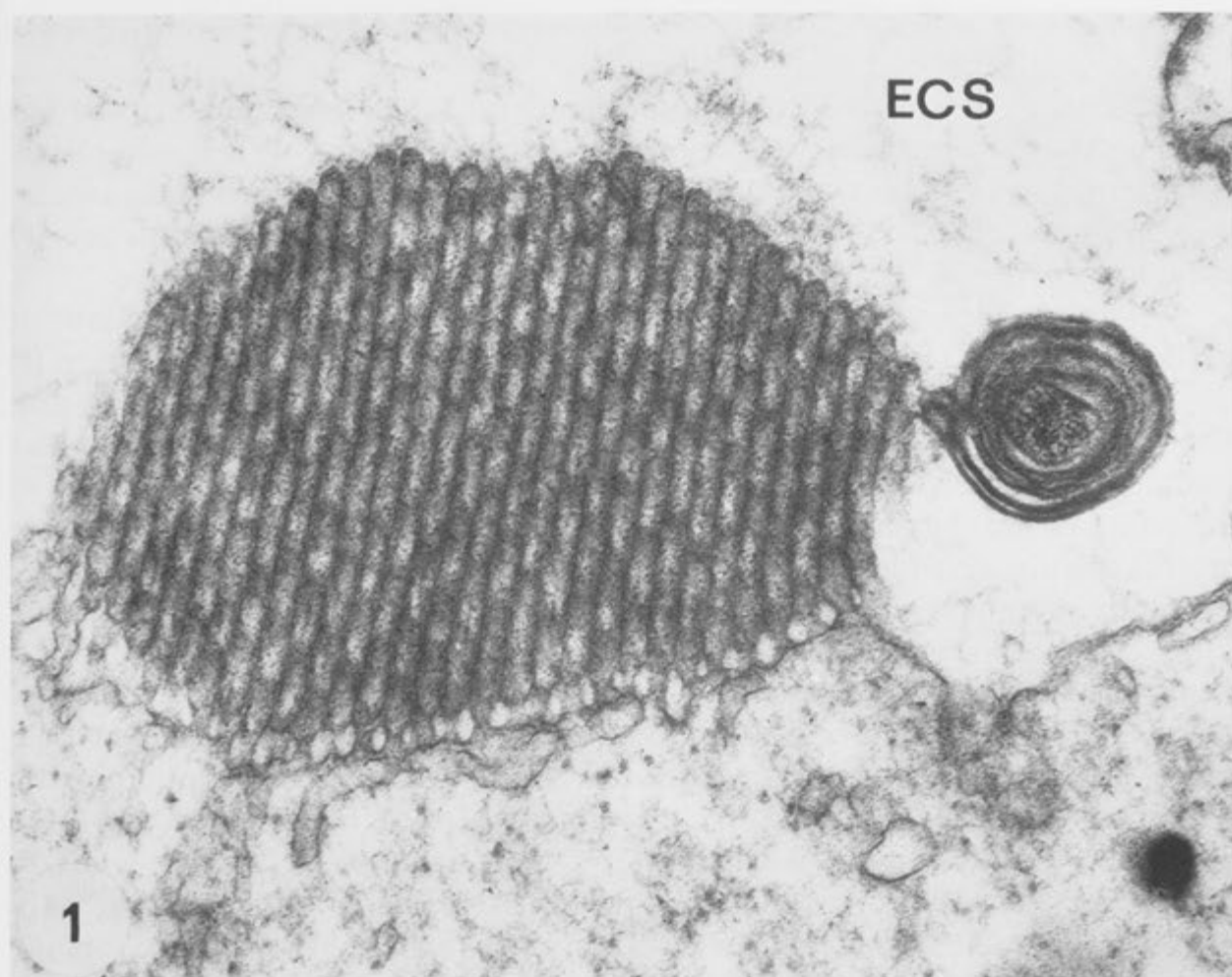
Comparisons were made between rhabdoms of *Ptilogyna* fixed for 1 h and 20 h, using fixatives 1 or 2 followed by a brief wash and osmication.

Subsequent Procedure. After fixation, tissues were washed in distilled water for 20–30 min, dehydrated in ethanol, and embedded in Araldite. Silver-grey sections were cut with glass knives, and poststained in saturated aqueous uranyl acetate for 30 min and Reynolds' (1963) lead citrate for 15 min. They were examined on a Jeol 100C or Hitachi H500 electron microscope.

Results

Blowfly

Description of Artefactual Structural Transformations. The fly retina consists of an array of open rhabdoms. In each rhabdom, rhabdomeres from eight reticular cells project into a large central extracellular space (ECS). A rhabdomere consists of precisely aligned photoreceptive microvilli, each connected to its receptor cell by a narrow neck (Figs. 1, 2) (see Trujillo-Cenóz and Melamed, 1966, for a detailed description).



Figs. 1 and 2. Whorls attached to the lateral margins of the rhabdomeres in *Lucilia*. Fixed for 20 h in 2.5% glutaraldehyde in cacodylate buffer with 2 mM CaCl_2 prior to osmication. The whorl in Fig. 2 was poststained longer in lead citrate than that in Fig. 1. ECS extracellular space. Fig. 1: $\times 56,000$; Fig. 2: $\times 66,000$

Fig. 3. Unattached whorl in extracellular space in the centre of a rhabdom in *Lucilia*. Fixed for 20 h in 2.5% glutaraldehyde and 2% formaldehyde in phosphate buffer with 0.9 mM CaCl_2 prior to osmication. $\times 150,000$

The artefacts are formed from photoreceptor microvilli that have collapsed and rearranged to form sheets of membrane, which roll up. When completely transformed, the resulting "myelin figure", or "whorl", is organised like a loosely arranged spiral cylinder, so that even in its centre the rolled-up membrane usually has a low curvature.

Whorls were found attached to rhabdomeres: they were observed (i) mostly at the lateral margins (Figs. 1, 2), often appearing to be peeling off into ECS (Fig. 1); (ii) occasionally at the microvillar bases, and (iii) only rarely at the microvillar tips. They were found in the centres of the rhabdomeres only after fixation in buffer containing 10 mM CaCl_2 . Unattached whorls were frequently seen in the ECS in the centre of the rhabdom (Fig. 3), and sometimes inside receptor cells. In some profiles whorls were observed as if in the process of being endocytosed from the ECS into a retinular cell by pseudopodia (Fig. 4).

Conditions for Occurrence. After fixation by fixatives 1 and 2 (see Materials and Methods), whorls were present in all samples that had been stored for 20 h before osmication, and in most samples stored for 7 h. There was no apparent difference between samples kept in fixative for long periods and only briefly washed, and those fixed for 1 h and then washed in buffer for the same total duration prior to osmication. Whorls were not found in material that was (a) osmicated only, (b) fixed in a glutaraldehyde-osmium tetroxide mixture (fixative 3); or (c) fixed for only 1 h in either fixative 1 or 2 prior to immediate osmication. With these three methods some segments of microvilli appeared to have detached from their rhabdomeres, and were visible in the ECS around some rhabdomeres as very lightly stained tubular structures (Fig. 5). However, their detachment might not have been artefactual (see Discussion). Of these methods a-c, c gave a far superior quality of preservation.

Samples left for 20 h in fixative 1 with different concentrations of CaCl_2 showed varied degrees of artefactual disfiguration. With 10 mM CaCl_2 , rhabdomeres were often badly disrupted by large whorls forming throughout them. Lower concentrations of CaCl_2 resulted in less severe artefact formation. In samples fixed with 0.5 mM CaCl_2 , only rings of membrane, considered to be early forms of whorls and stained less darkly, were seen at the lateral margins of rhabdomeres. A few of these 'rings' of membrane were evident in samples fixed with no CaCl_2 , but were only particularly noticeable after fixation for 3 days prior to osmication. Fixation with buffer containing 2 mM EGTA, even for 3 days, produced no signs of whorl formation whatsoever; the retinular cells resembled material fixed in fixatives 1 or 2 for only 1 h prior to osmication, and their quality of preservation was as good.

Tipulid: Whorl Formation in Shedding Zone

For the purpose of this account the rhabdom of the tipulid *Ptilogyna* may be considered as an open rhabdom similar to that of *Lucilia* (Williams, 1980).

With fixatives 1 or 2, whorls, smaller and less distinctive than those in *Lucilia*, were found in the rhabdom of *Ptilogyna* after 20 h-fixation, but not after 1 h. They were restricted to the transformed distal segments of microvilli, which have been shown to be in the process of shedding during photoreceptor membrane turnover

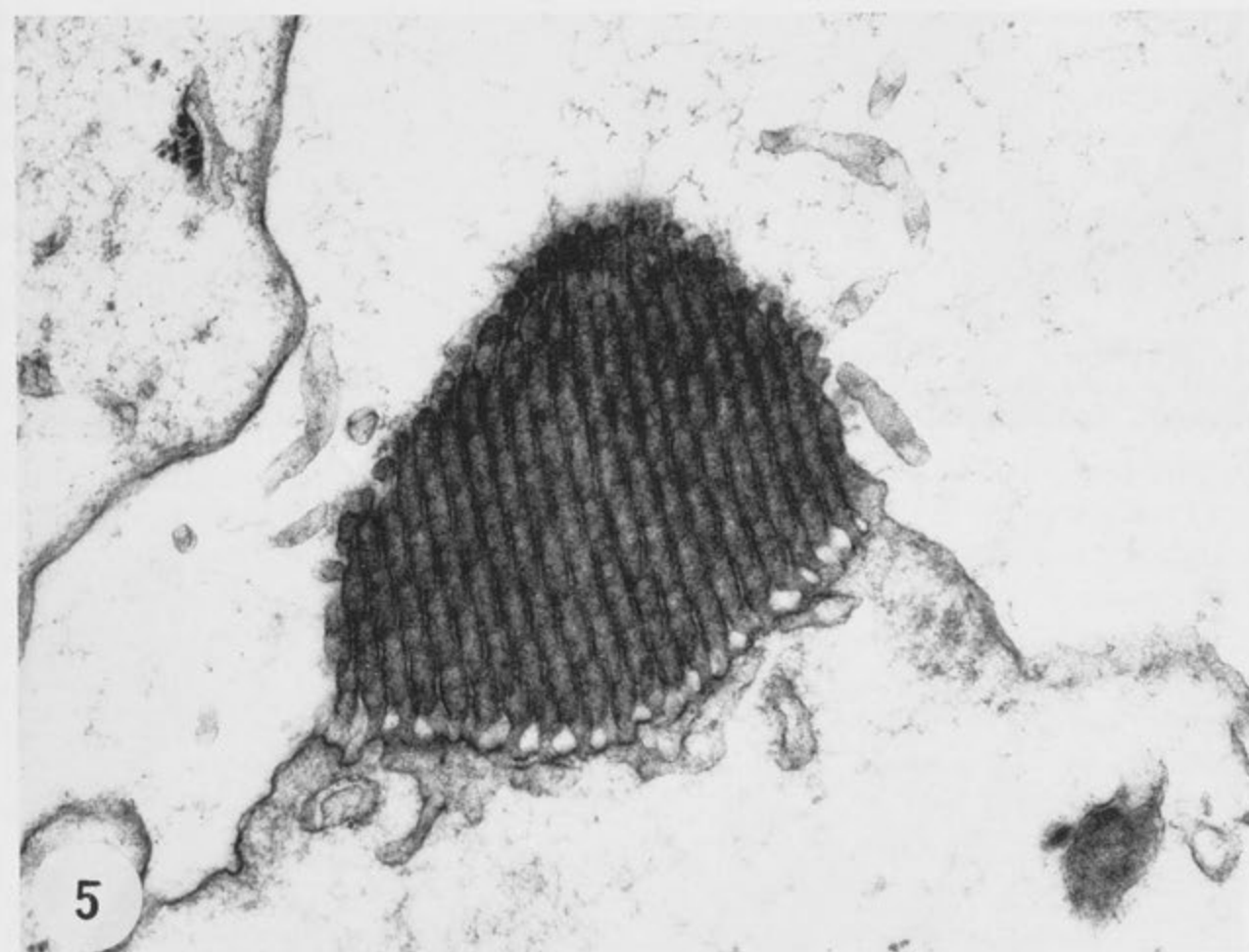


Fig. 4. Whorl in *Lucilia* being endocytosed by a pseudopodium into a reticular cell from extracellular space. Fixed for 7 h in 2.5% glutaraldehyde and 2% formaldehyde in phosphate buffer with 0.9 mM CaCl_2 prior to osmication. $\times 65,000$

Fig. 5. Rhabdomere in *Lucilia* fixed for 1 h in 2.5% glutaraldehyde in cacodylate buffer with 2 mM CaCl_2 prior to osmication. The very lightly stained tubular structures in extracellular space around the rhabdomere are possibly segments of microvilli shed during normal photoreceptor membrane turnover. $\times 60,000$

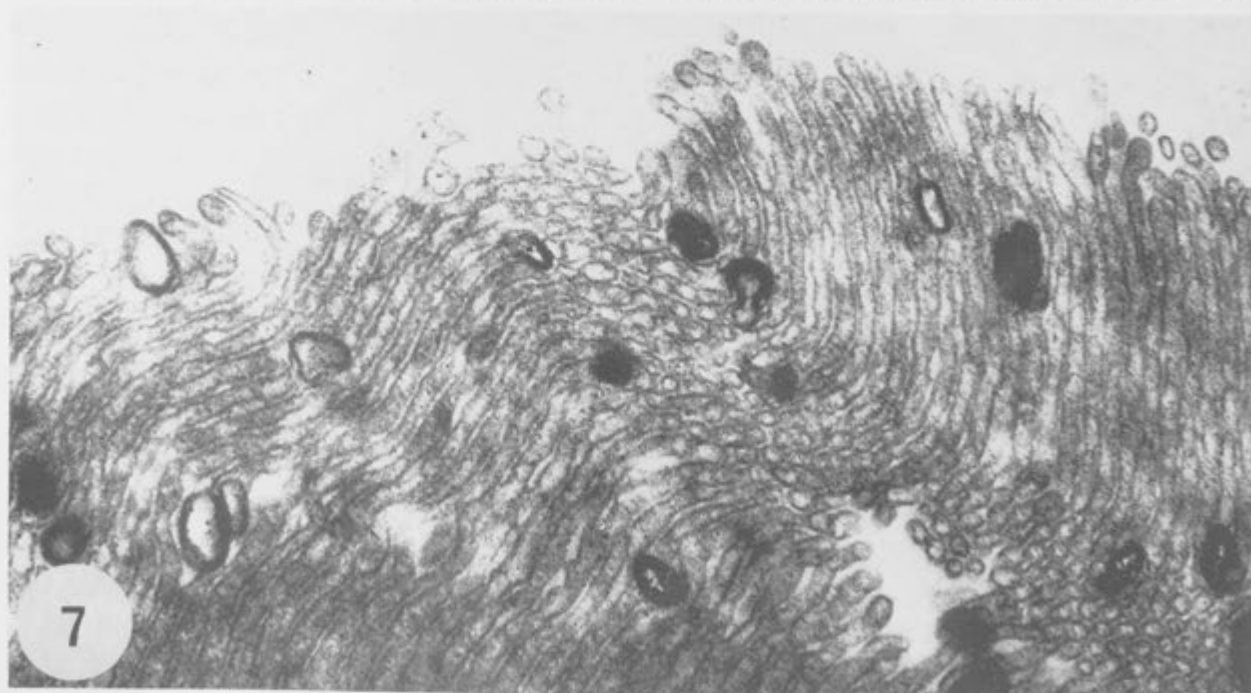
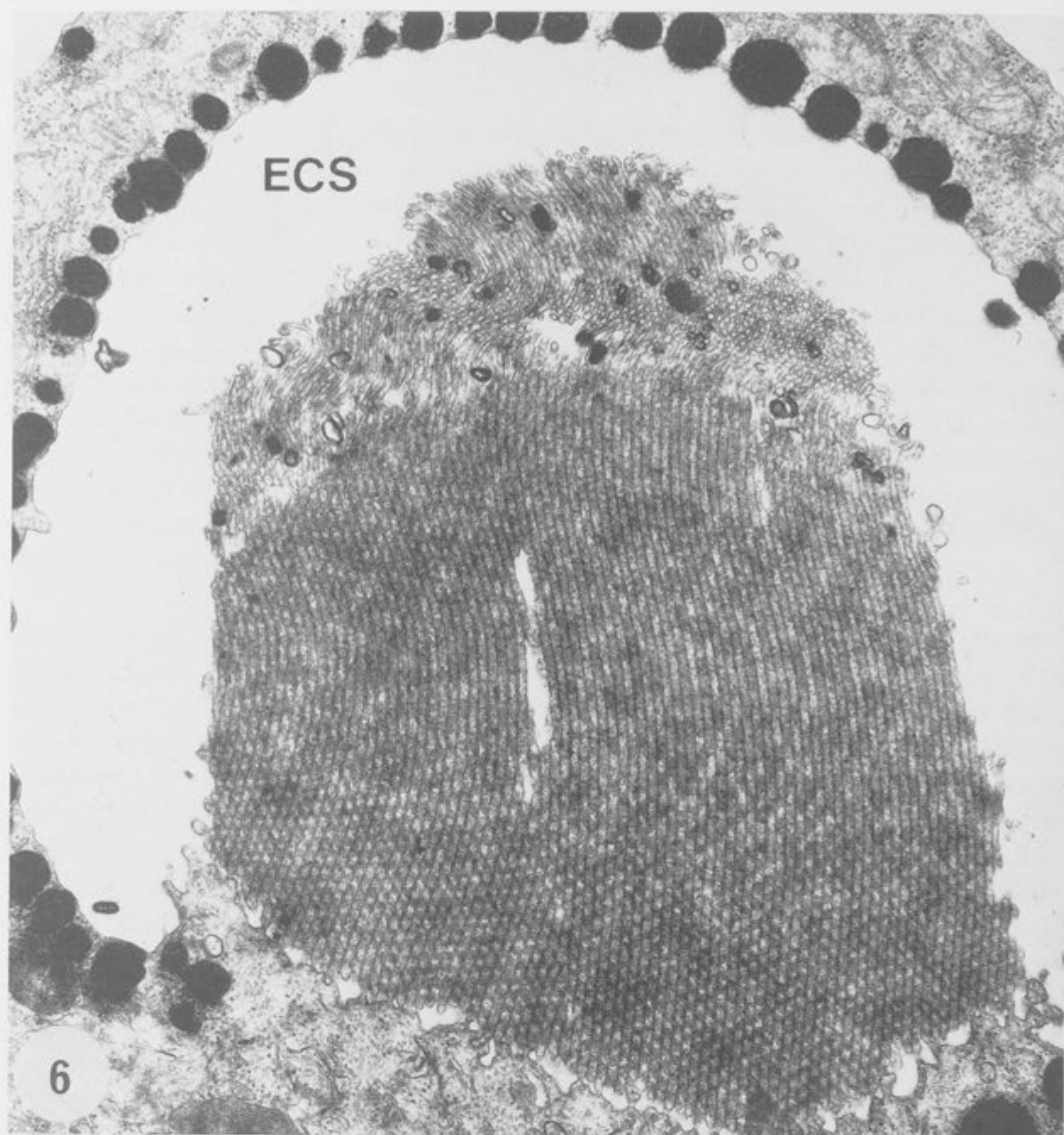


Fig. 6. Rhabdomere of *Ptilogyna* fixed for 20 h in 2.5% glutaraldehyde and 2% formaldehyde in phosphate buffer with 0.9 mM CaCl_2 prior to osmication. Small whorls are present in the shedding zone. ECS extracellular space. $\times 24,000$

Fig. 7. Higher magnification of part of the shedding zone in Fig. 6. $\times 60,000$

(Williams and Blest, 1980). Very occasionally, whorls were also observed at the bases of the microvilli.

Discussion

The present results show that drastic alterations of photoreceptor microvilli occur if tissues are left too long in conventional Ca^{++} -containing buffers before osmication, and thus illustrate the dangers of the common practice of leaving tissues "overnight" in primary fixative or wash. Moreover, the formation of artefactual whorls might not be restricted to photoreceptor microvilli, since similarly structured "myelin figures" have been reported adjacent to plasma membrane in numerous other types of tissues (e.g., Sabatini et al., 1963; Trump and Ericson, 1965).

While glutaraldehyde is well known for its superior ability to crosslink proteins, its reaction with lipids is minimal (Bowes and Cater, 1966). Polymerisation of lipids occurs during osmication (Korn, 1967). Prior to osmication, therefore, tissues are vulnerable to lipid extraction. It is possible that the photoreceptor microvilli lose some lipids during primary fixation in glutaraldehyde or subsequent wash in buffer, and consequently become more susceptible to the effects of calcium ions. It is not known how Ca^{++} induces the transformation of microvilli to whorls, but it might be similar to the manner in which Ca^{++} disrupts laboratory-prepared phospholipid vesicles and subsequently fuses them into "cochleate lipid cylinders" (Papahadjopoulos et al., 1975). The latter are similar to the whorls depicted here, only more ordered, and both the phospholipid vesicles used by Papahadjopoulos et al., and photoreceptor microvilli are characterised by small radii of curvature.

Not all photoreceptor membrane transformed into whorls with equal readiness. Except with buffer containing 10 mM CaCl_2 , whorls formed in *Lucilia* only at the periphery of a rhabdomere, mainly from the lateral microvilli. In *Ptilogyna*, whorls formed nearly exclusively in the transformed shedding zone (Williams and Blest, 1980) at the distal ends of the microvilli. It is tempting, therefore, to suggest on comparative grounds that many of the whorls in *Lucilia* were formed from shedding microvillar membrane, and that the very lightly stained unattached segments of microvilli observed around some rhabdomeres in whorl-free samples (Fig. 5) were detached from the lateral margins of the rhabdomere during this natural process. If this is so, the observed uptake of whorls by reticular cells from ECS (Fig. 4) implies that in *Lucilia* at least some photoreceptor membrane follows an extracellular shedding route like that described in *Ptilogyna* by Williams and Blest (1980).

The whorls that occasionally formed at the bases of the microvilli in *Ptilogyna* and *Lucilia* might also be formed from membrane about to be shed, since in both flies some membrane material is shed by basal pinocytosis (Williams and Blest, 1980; own unpublished observations). Alternatively, their formation here might be due to a different structure of the microvilli at their bases, making their necks more susceptible to disruption by Ca^{++} .

Whorl-like bodies have also been described in the photoreceptor microvilli of the crayfish, *Procambarus* (Roach and Wiersma, 1974). These authors left their tissues "overnight" in phosphate buffer, so that the whorls they reported were

probably caused in the same way as the artefact described here, although they do not mention whether or not their buffer solutions contained Ca^{++} . Interestingly, the number of whorls appeared to decrease with the length of time the rhabdoms has been maintained in darkness (for up to 11 weeks) prior to fixation. In *Procambarus* (as well as in all other invertebrates so far tested), photoreceptor membrane shedding slows down if animals are kept in prolonged darkness (Eguchi and Waterman, 1979). Therefore, the decrease in whorl numbers noted by Roach and Wiersma would have coincided with a decrease in the amount of photoreceptor membrane shedding. Accordingly, evidence from *Procambarus* and the two flies, *Lucilia* and *Ptilogyna*, suggests that changes related to shedding of photoreceptor microvillar membrane increases the facility with which the microvilli are disrupted by Ca^{++} to form whorls during processing prior to osmication.

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CHAPTER V

Note: in this chapter "retinula" refers to a single retinular cell.

Extracellular Shedding of Photoreceptor Membrane in the Open Rhabdom of a Tipulid Fly

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Summary. The compound eyes of the Australian tipulid fly, *Ptilogyna*, shed the bulk of their rhabdomeral membrane to extracellular space during turnover. The rhabdomeres of the retinulae lie in a common extracellular space (ECS), which is subdivided in the proximal retina. Before dawn, a distal region of the microvilli in each rhabdomere differentiates and becomes less electron-dense after conventional fixation. The differentiated region then dilates and develops an irregular profile. A few hours after dawn, the transformed tips break off and form a detritus in the ECS. The degraded membrane is internalised back into the retinula cells by mass endocytosis. Retinulae develop pseudopodia at sites bordering the ECS and engulf the membrane detritus, which comes to lie first of all in vacuoles within the receptor cells and then forms very large multivesicular bodies. The latter transform to multilamellar and residual bodies and are, presumably, lysed. Surrounding these secondary lysosomes are rough endoplasmic reticulum and smooth tubular systems, tentatively considered on comparative grounds to provide hydrolases. The literature concerning the ultrastructure of compound eyes offers a small number of instances where extracellular shedding can be suspected for morphological reasons. Attention is drawn to analogies with the shedding of photoreceptor membranes in vertebrate retinæ.

Key words: Open rhabdom – Microvillus – Extracellular membrane shedding – Pseudopodial uptake – Secondary lysosomes – Fly, *Ptilogyna spectabilis* (Skuse).

Photoreceptors of the anterior lateral eyes of the salticid spider, *Plexippus*, shed membrane from the microvilli of their rhabdomeres to extracellular space (ECS),

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from which it is retrieved by processes of the nonpigmented glia (Blest and Maples, 1979). Previously, a similar route of shedding had been suspected to be implicated during turnover of rhabdomere membrane in another spider, *Dolomedes* (Blest and Day, 1978). The former authors suggested that an extracellular route might explain the apparent absence of products of membrane degradation in the receptor cytoplasm of flies with open rhabdoms (Melamed and Trujillo-Cenóz, 1968; Boschek, 1971). Preliminary examination of receptors of the blowfly, *Lucilia*, has not so far resolved this question, although intracellular shedding via pinocytosis has been shown to take place (Williams, in preparation).

In the present report, massive extracellular shedding in the retina of a tipulid fly is described, and it is shown that the receptors retrieve products derived from the shed membrane by endocytosis from the ECS.

Materials and Methods

Both sexes of the large crepuscular and nocturnal Australian tipulid *Ptilogyna* (*Plusiomya*) *spectabilis* (Skuse) (Dobrotworski, 1971) were collected at Captain's Flat, Karwarrie, and at Braidwood, both in New South Wales. Some specimens were fixed at the site of capture, but most were conveyed to the laboratory in Canberra in plastic bags resting on ice in a polystyrene container with a window to sustain the normal daily cycle of illumination. They were then stored in a room exposed to daylight which was well-diffused to prevent exposure to direct sunlight. The temperature of the room was not controlled other than to avoid heating above ambient environmental temperatures and to avoid cooling below 20°C at night. The range of temperatures experienced by these flies was probably narrower than that of the external environment. Flies were fixed for electron microscopy at various times throughout the 24 h light cycle up to 48 h after capture; some were also sampled after being held in the dark for up to three days and then exposed to various durations of natural daylight.

Retinae were dissected so as to expose tissue to the fixative as rapidly as possible. The nature of the effects described below means that very rapid fixation is important; Griffiths and Boschek (1976) and Griffiths (1979) have shown that significant ultrastructural changes of the fly retina are evident as soon as 3 min after injury to the receptors. Flies were decapitated and the lateral margins of the eyes trimmed with microcissors under primary fixative at 4°C so that only a small block of the central retina remained. The whole dissection was completed in less than 1 min, and the procedure was performed under light filtered by Schott RG 665 (red cut-off) and KG3 (heat) filters. Room lighting was either on or off to coincide with day or night.

Primary fixatives were: (a) 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.3 with 0.07 or 0.14 M sucrose and 2 mM CaCl_2 ; (b) 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium dihydrogen orthophosphate-NaOH buffer at pH 7.3 with 0.07 M D-glucose and 0.9 mM CaCl_2 ; (c) 2.5% glutaraldehyde in 0.1 M PIPES-NaOH buffer at pH 7.3 with 0.07 or 0.14 M sucrose and 2 mM CaCl_2 .

The best results were obtained with fixatives (a) and (b); 0.07 M sucrose provided the better tonicity. After a brief wash, tissues were post-fixed for 0.5–1 h in 1% OsO_4 in the same buffer. They were embedded in Araldite, and grey to silver-grey sections were collected on formvar-coated slot grids, or on 400-mesh grids without a supporting film, and examined in a Jeol 100C or Hitachi H500 electron microscope.

Results

General Anatomy

A full account of the dark- and light-adapted eye will be given separately (Williams, in preparation). The gross anatomy of the retinae of five genera of

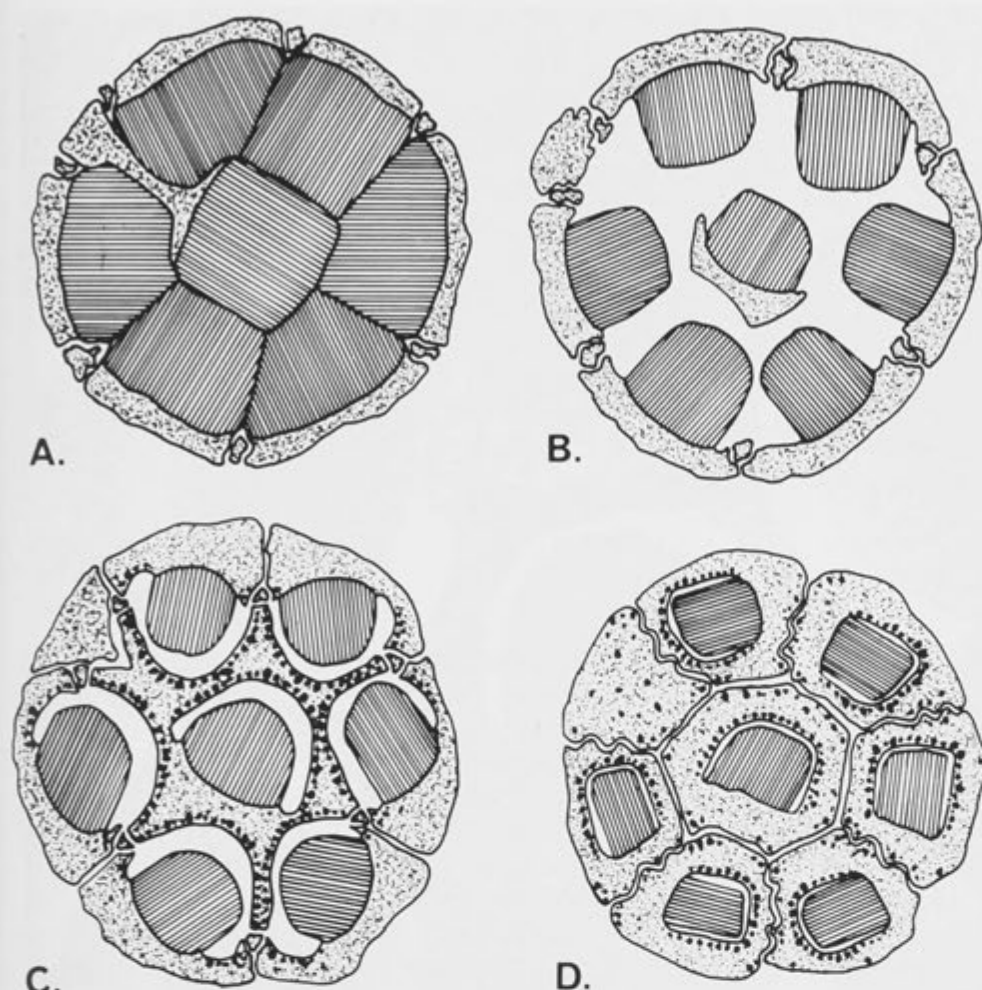


Fig. 1. Diagram illustrating the four different regions of the rhabdom in transverse section. A–D are profiles taken at increasing distances from the cornea

European Tipulidae was described by Sotavalta et al. (1962), on the basis of light microscopy alone. At that level of resolution our results agree basically with those of Sotavalta et al., but the latter did not resolve an eighth cell and concluded that each ommatidium possessed seven retinulae only.

The organisation of a single ommatidium is illustrated in Figs. 1 and 2. Figure 1 shows a series of transverse sections taken at different levels. Distally, beneath the crystalline cone, the rhabdomeres of six retinulae (R1–6) are contiguous with each other laterally, and centrally with the short rhabdomere of R7. There is no large ECS, resulting in a distal fused rhabdom composed of the rhabdomeres of R1–7 (Fig. 1A). Beneath this region, there is a short segment of the ommatidium with a large common ECS. Here, the rhabdomeres of R1–6 are for the most part separated, and the central rhabdomere of R8 lies free of them (Fig. 1B). More proximally, processes from R8 extend around its own rhabdomere and laterally to the margins of the rhabdom to form the tops of “pockets”, in which the proximal parts of the rhabdomeres of R1–6 and 8 lie (Fig. 1C and 2). In the basal part of the rhabdom the lateral processes of R8 are replaced by extensions from R1–6 so that each retinula encloses its own rhabdomere and continues the “pocket” (Fig. 1D). The seven pockets, which appear in transverse section to contain small isolated ECSs around individual rhabdomeres, are thus connected to the large common central ommatidial ECS in the distal rhabdom.

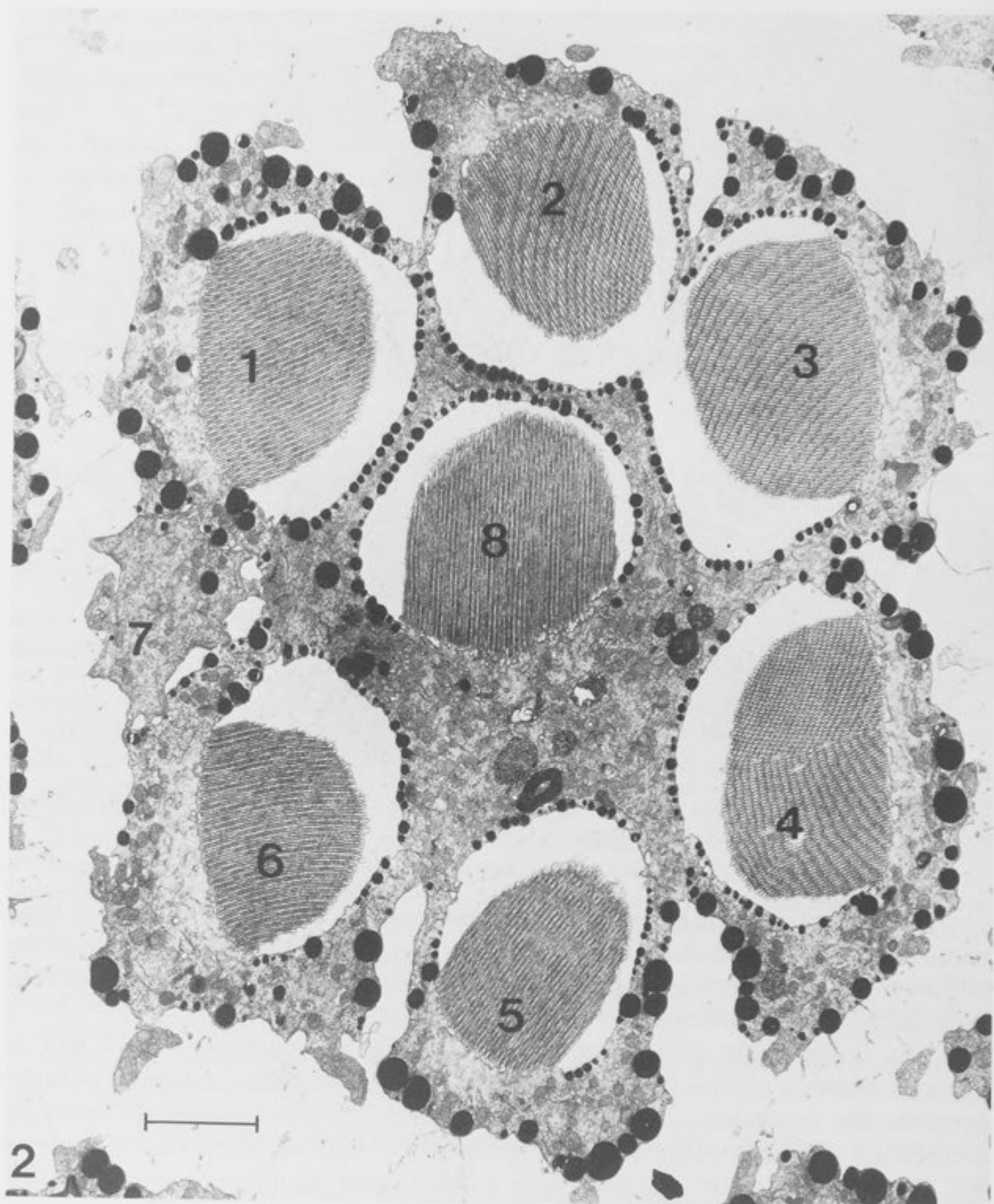


Fig. 2. Approximately transverse section of an ommatidium fixed in the afternoon. The top portion of the profile is slightly more distal than the bottom. The section samples the distal part of the region where the rhabdomeres lie in "pockets" formed by extensions of the retinulae. Retinulae are numbered. $\times 7,000$. Scale = $2\mu\text{m}$

Each rhabdomere of R1-6 twists down its length in a manner that cannot be artefactual, a feature that will be discussed later (Williams, in preparation). The rhabdom is short ($50\mu\text{m}$) occupying only half of the distance between the lenslet and basement membrane. The bulk of the cytoplasm of the retinulae, together with their nuclei, lie proximal to the rhabdom. The retinulae contain pigment granules, and the assembly of retinulae is surrounded by pigment cells.

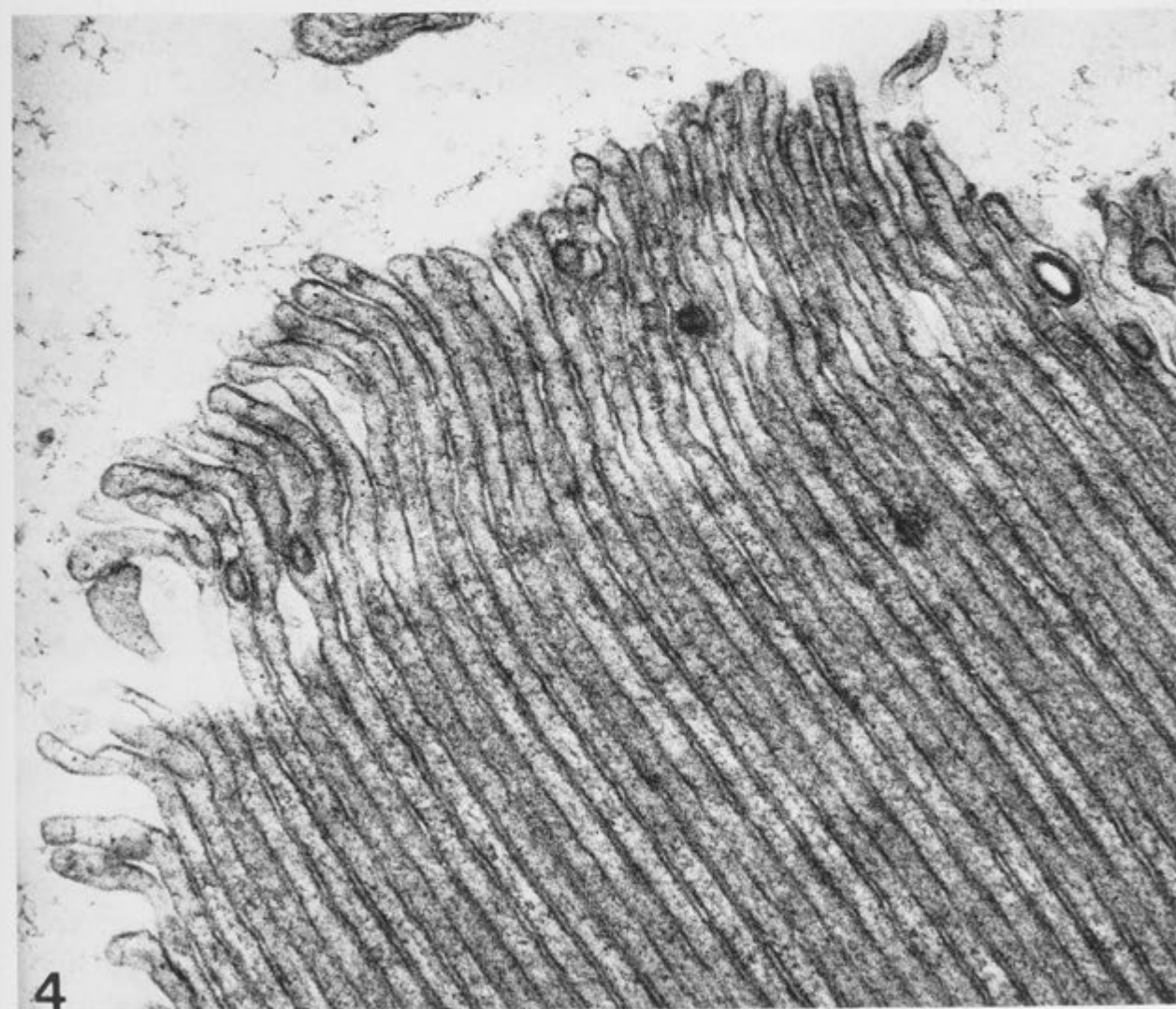
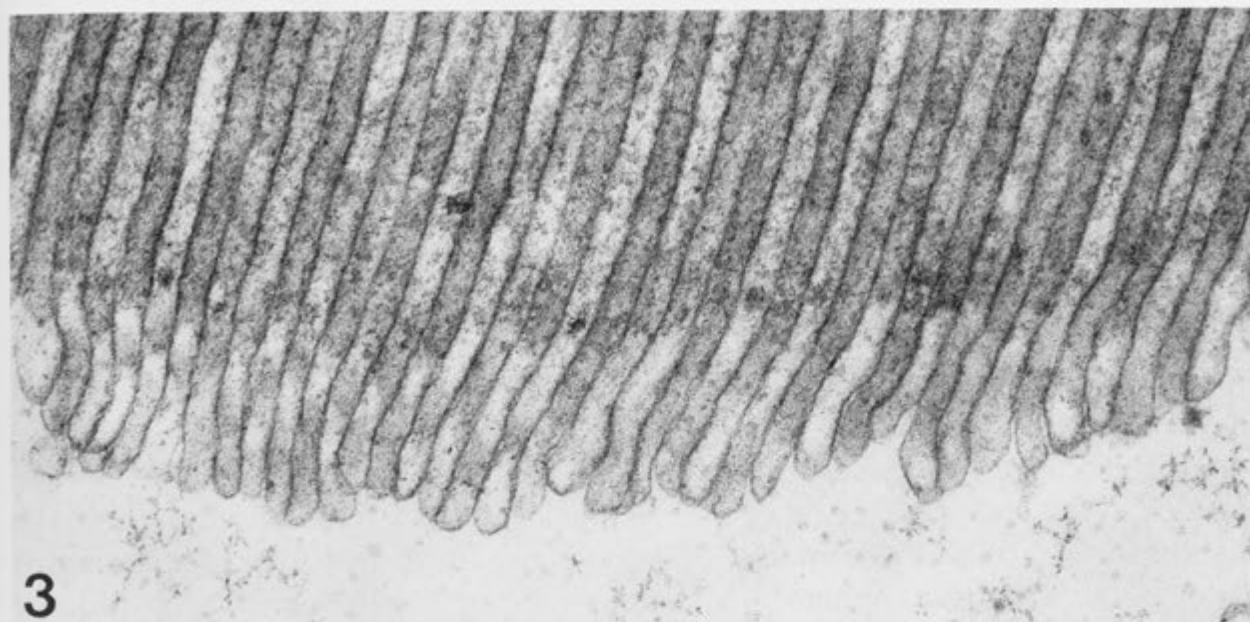


Fig. 3. Microvilli from a rhabdomere of an eye fixed immediately before dawn. Their tips have become less electron-dense and are irregular in profile. $\times 54,000$

Fig. 4. A slightly later stage than Fig. 3. The tips of the microvilli are more disorganised. $\times 54,000$

The shedding of distal segments of microvilli into ECS, described below, occurs in all parts of the rhabdom, including the fused region.

Extracellular Shedding of Distal Segments of Microvilli

Before dawn, the tips of the microvilli undergo a transformation. They become less electron-dense, dilate, and show signs of vesiculation (Figs. 3 and 4). After dawn these changes are exaggerated, and entire tissue fragments consisting of microvillus tips break off from their rhabdomeres to yield an irregularly vesicular detritus in the ECS (Figs. 5–7).

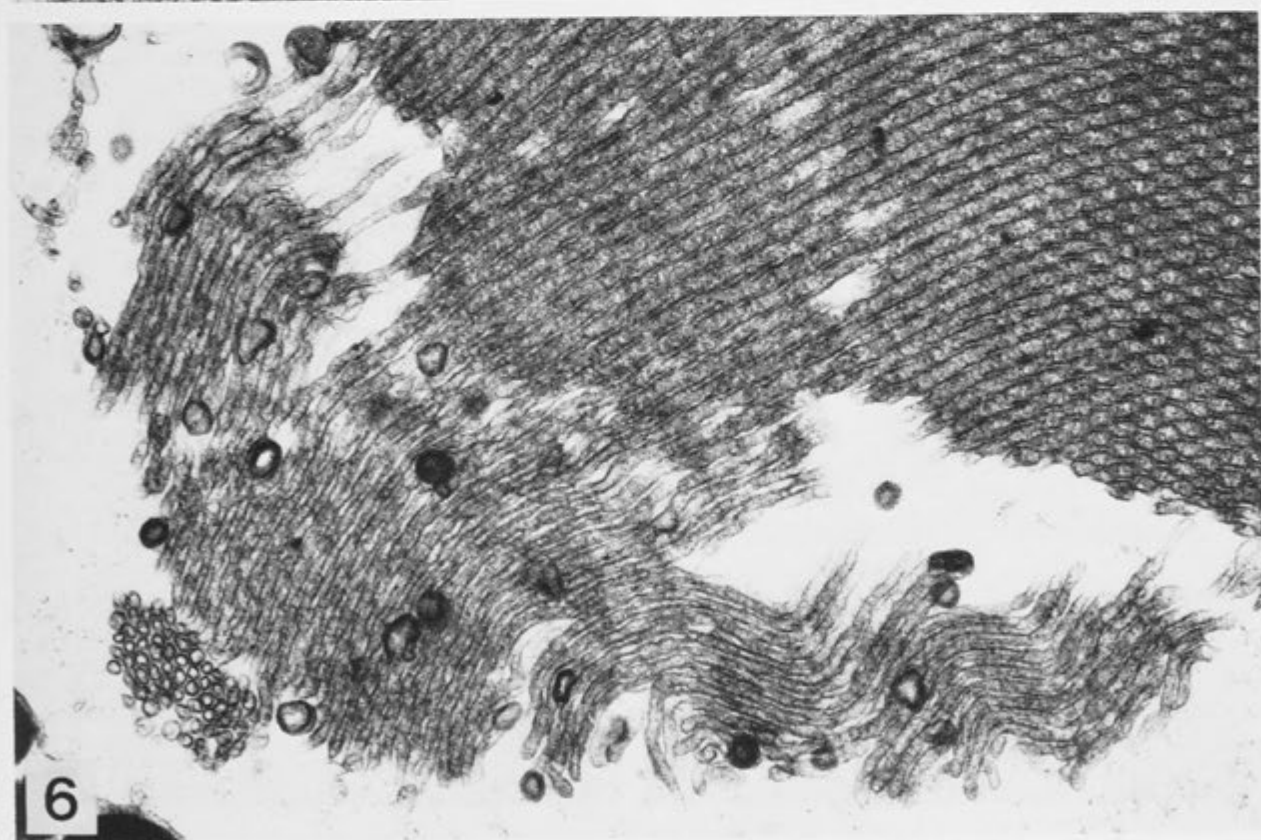
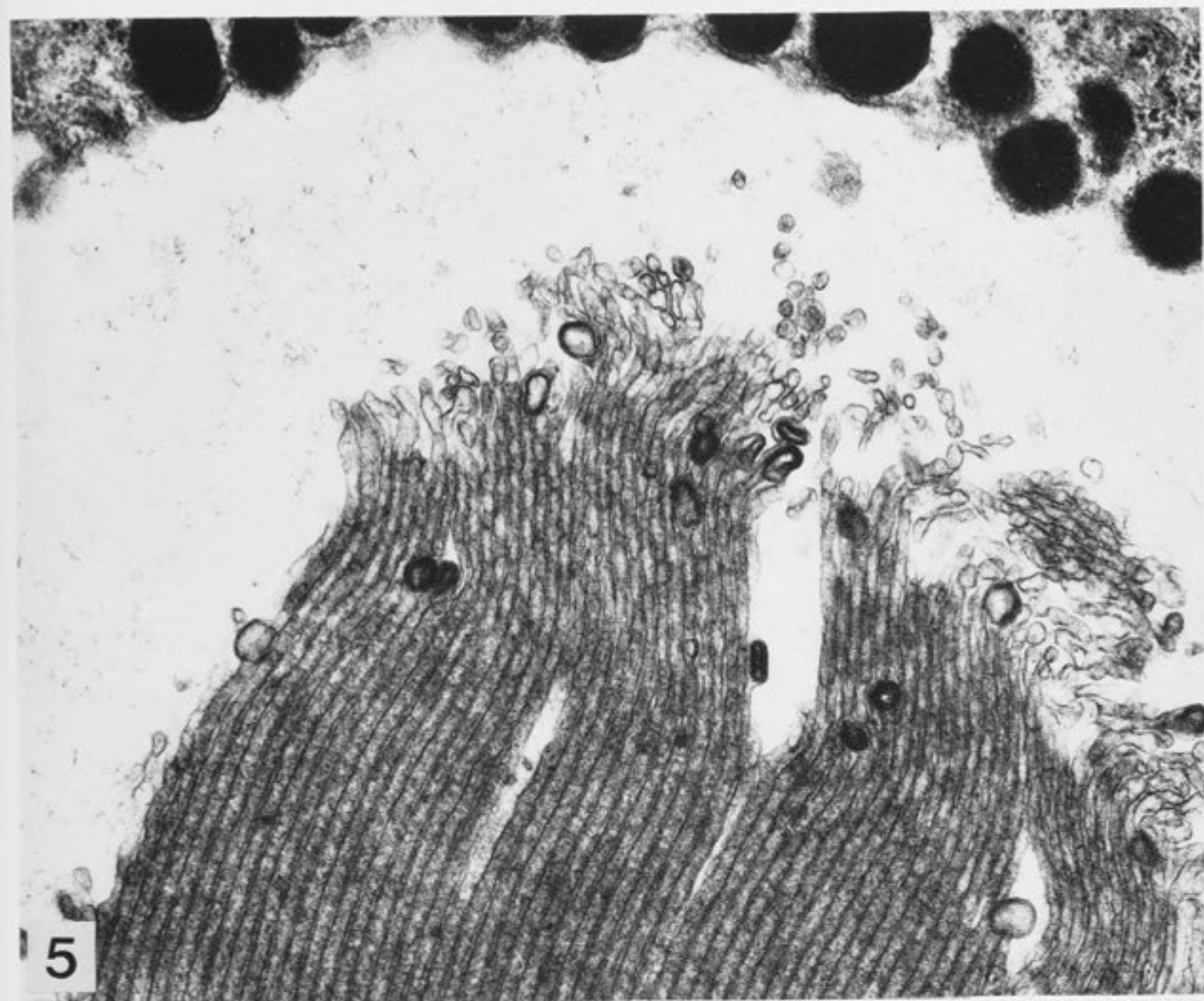
At this stage in the cycle, the state of the receptors implies that shed membrane is endocytosed back into the retinulae. The surfaces of the retinulae adjacent to the rhabdomeres acquire pseudopodial processes (Figs. 7, 8 and 10) and sparse coated endocytotic pits (Fig. 8). Vacuoles within retinulae now contain vesicles, profiles of which match those of vesicles in the ECS, and amorphous material, that appears similar to the material scattered throughout the ECS (Figs. 8 and 9). In the normal retina pseudopodial uptake is not readily observed, probably because it happens very rapidly. It is signalled most conspicuously by the presence of vacuoles in the retinulae. When, however, flies are held in continuous darkness for 2–3 days and then exposed to a normal dawn, very large masses of membrane are shed, and pseudopodial profiles (Figs. 7, 8 and 10) are readily observed.

Endocytosed membrane products eventually aggregate as multivesicular bodies (mvbs) in the cytoplasm of the retinulae around the rhabdom. By midday, very large mvbs are present (Fig. 14), and may be easily seen with the light microscope. Later in the afternoon, large vacuoles containing only a few vesicles are more abundant here (Fig. 11). This suggests that after midday the amount of membrane being shed decreases, but phagocytosis continues, taking up more extracellular fluid than membrane. Small residual bodies are found in retinulae in the morning and large residual bodies by the afternoon (Fig. 16). These secondary lysosomes presumably result from degradation of mvbs via multilamellar bodies (Figs. 15–17), similar to that described by White (1967, 1968), Eguchi and Waterman (1976), and Blest et al. (1978a).

From late morning until mid-afternoon, secondary lysosomes occupy a significant proportion of the cytoplasmic volume of the retinulae, and become surrounded by fields of smooth endoplasmic reticulum, tubules and vesicles. The latter probably contain acid hydrolases, for similar organelles derived from the endoplasmic reticulum and associated with mvb degradation have been described in retinae of a spider *Dinopis* (Blest et al., 1978b) and in the grapsid crab *Leptograpsus* (Blest et al., 1980). In both the crab and the spider (Blest et al., 1979), they have been shown to be acid phosphatase-positive, and have been discussed as a specialised GERL system.

Timing of the Cycle

The timing of events outlined above is not precise, although some generalisations can be made. Receptors sampled between midnight and 04.00 h lack secondary



Figs. 5 and 6. Microvilli from rhabdomeres of eyes fixed after dawn. The tips of the microvilli are more transformed than in Figs. 3 and 4, and have begun to detach from the rhabdomere. $\times 37,000$

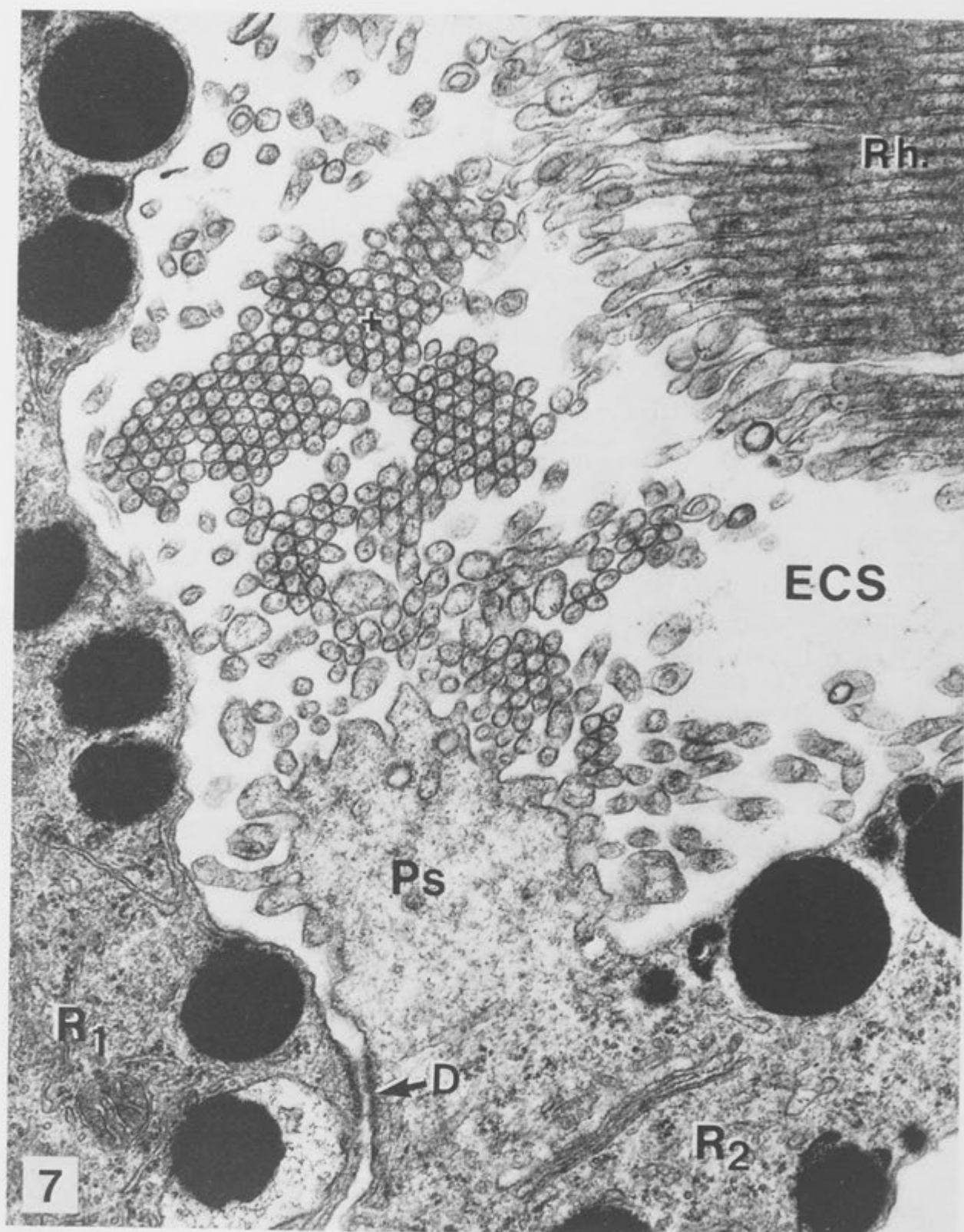
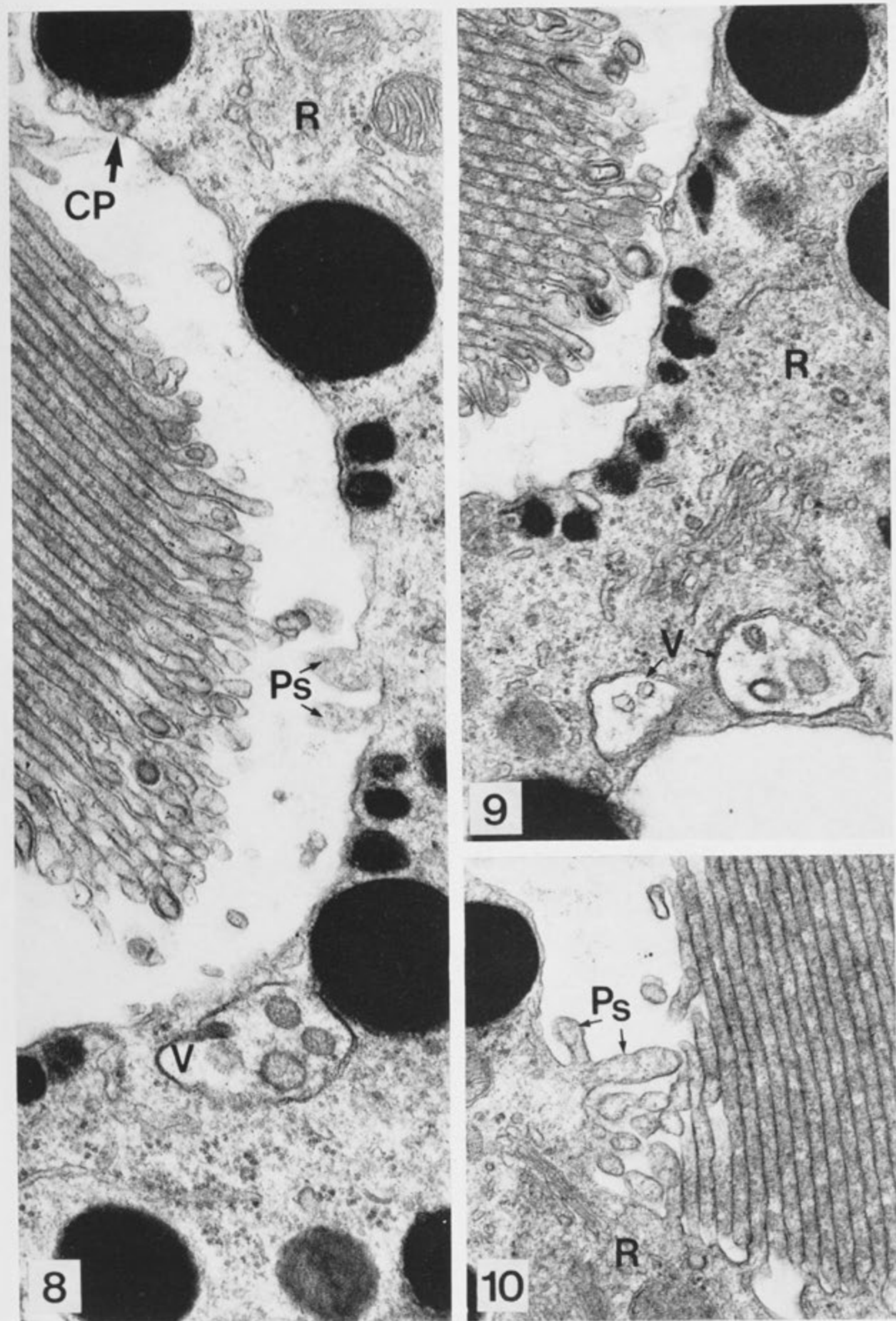


Fig. 7. Pseudopodia (*Ps*) extend from a retinula (*R2*) into the extracellular space (*ECS*) around a shedding rhabdomere (*Rh*). Some microvilli are in transverse section (+). They still show the organisation of intact microvilli, including axial filaments, and are probably still attached to the rhabdomere beyond the plane of section. *R1* retinula neighbouring *R2*; *D* desmosome at junction of *R1* and *R2*. $\times 43,000$



Figs. 8–10. Profiles of retinulae (*R*) with pseudopodia (*Ps*) projecting into ECS, and small vacuoles (*V*) containing vesicular profiles considered to be derived from shed microvilli. *Cp* coated pit. Fig. 8, $\times 47,000$; Fig. 9, $\times 44,000$; Fig. 10, $\times 39,000$

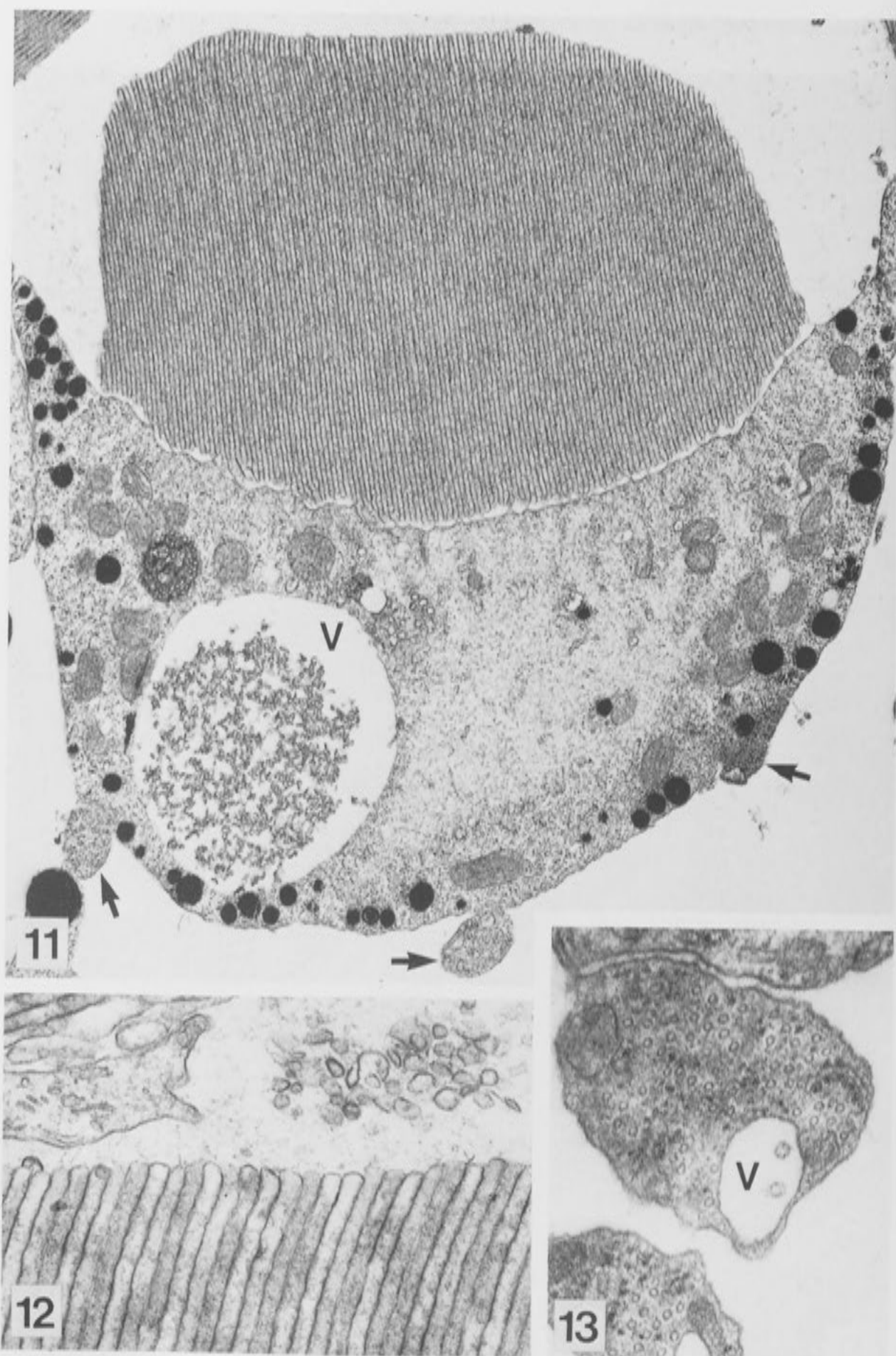


Fig. 11. Retinula fixed in the afternoon. The tips of the microvilli are orderly. The cytoplasm contains a large vacuole (*V*) only partially filled with vesicles derived from shed photoreceptor membrane. Arrows indicate secondary pigment cells. $\times 13,000$

Fig. 12. Distal tips of microvilli fixed in the afternoon. $\times 35,000$

Fig. 13. Transverse section of columns of secondary pigment cells in the region of the rhabdom. Vacuole (*V*) contains two vesicles. $\times 57,000$

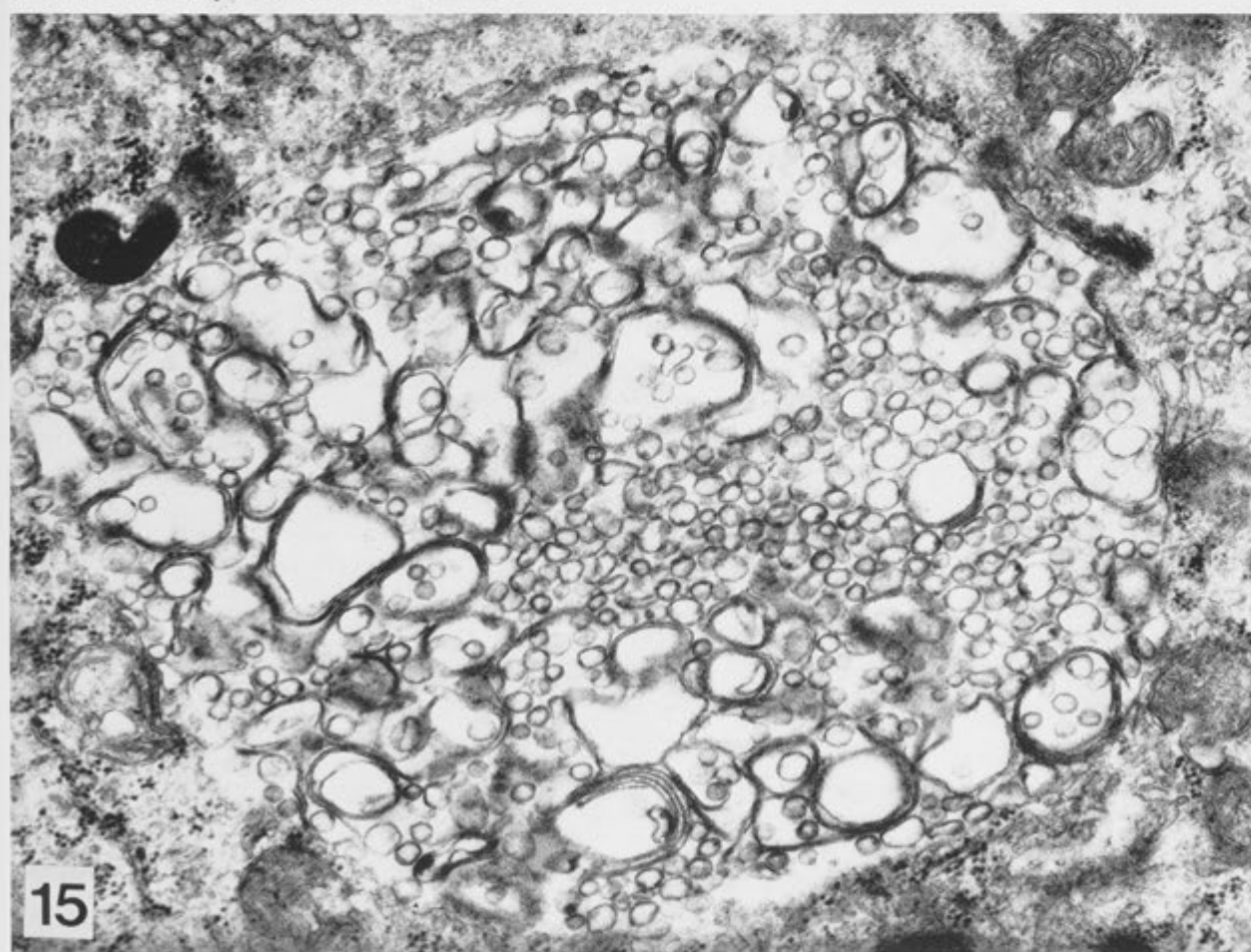
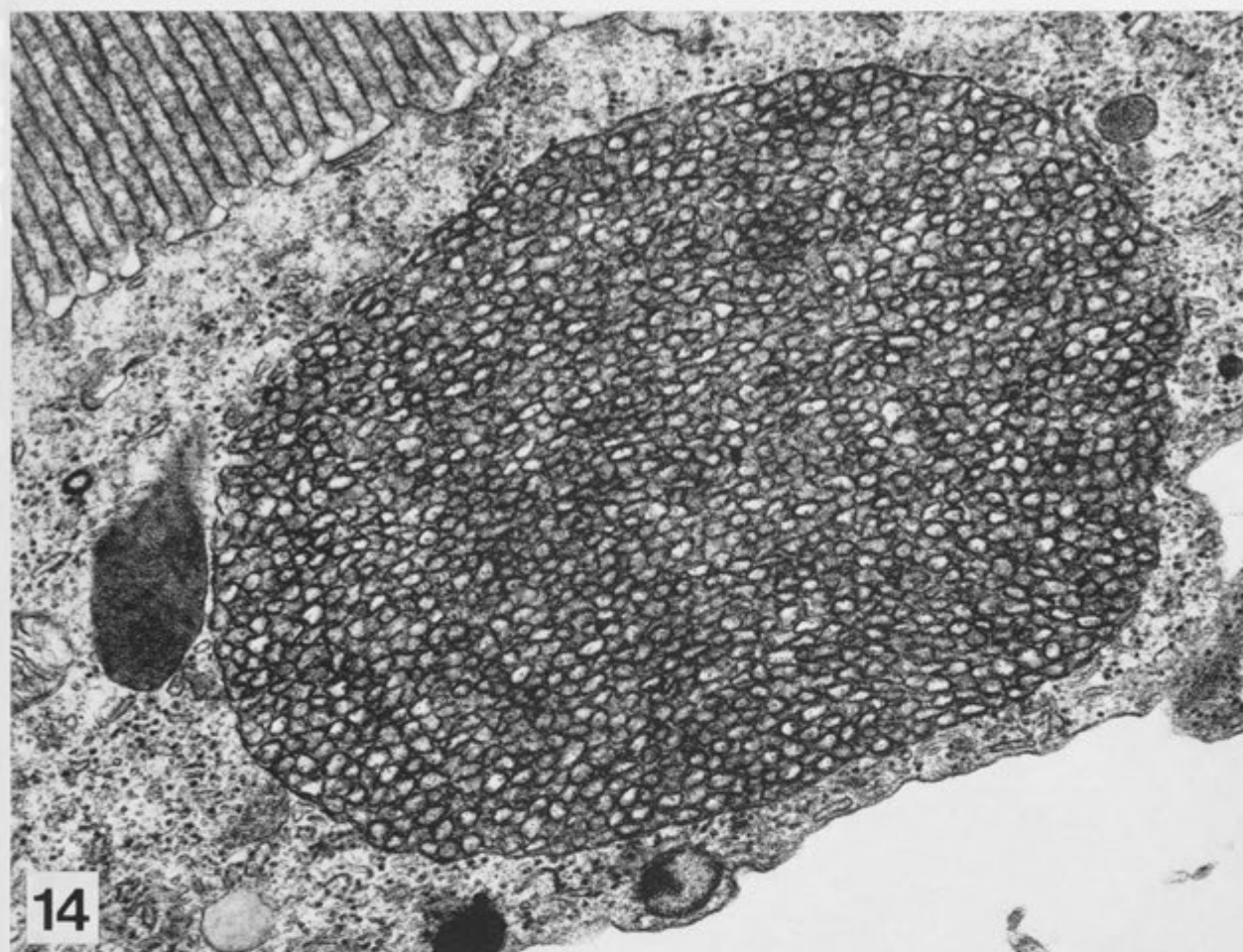


Fig. 14. Large, tightly-packed multivesicular body in retinula fixed at midday. $\times 36,000$

Fig. 15. Multivesicular body at later stage than Fig. 14. Vesicles are reorganising to form lamellae. $\times 36,000$



Fig. 16. Secondary lysosomes (*L1* and *L2*) in more advanced states of degradation than shown in Fig. 15. In *L1* vesicles have reorganised to multilamellae bodies. *L2* represents a later stage, consisting of multiple layers of phospholipid lamellae, some of which have partially coalesced. *G* secondary glial cell; *Ep* endocytotic pit. $\times 29,000$

Fig. 17. Parallel lamellae in a secondary lysosome at a stage slightly prior to *L2* in Fig. 16. $\times 190,000$

lysosomes, and the tips of their microvilli were intact and untransformed (Figs. 11, 12). All receptors fixed between dawn and mid-morning were found to be in the process of shedding. Between 12.00 and 16.00 h some cells had completed shedding to the point shown in Fig. 11, but the majority had not. Small mvbs are present in the retinulae by 2 h after dawn and residual bodies soon after; however, it is only during the middle of the day that mvbs of the massive size depicted in Fig. 14 appear. It can be concluded that shedding begins just before dawn, continues throughout the day, and has a pronounced peak around mid-morning. Degradation of membrane products probably begins in earnest after midday.

Shedding as a Normal Process

Shedding was established as a normal process by dissecting and fixing *Ptilogyna* in the field. Rhabdomeres were in identical stages of the cycle for given times of day as were those sampled after being held for up to 2 days in storage in Canberra.

Participation of the Secondary Pigment Cells in Membrane Uptake

The common ECS around the rhabdomeres of a single ommatidium appears to be isolated from the ECS outside the ommatidium by the juxtaposition of R1-7 to their neighbours (Fig. 2; Williams, in preparation); often there is a desmosome at the junction two neighbouring cells (Fig. 8). Therefore, there is little to suggest that material can leak from the central ECS to the extra-ommatidial ECS. Nevertheless, columns of secondary pigment cells outside the central region of the ommatidium can be seen to possess endocytotic pits (Fig. 16) and occasionally small vacuoles, which may contain vesicles or other less well-defined material (Fig. 13). Profiles of receptors sometimes suggest that the retinulae may produce small pseudopodia into the extra-ommatidial ECS. If these pseudopodia were to break off and be endocytosed by the pigment cells, this might account for the fragments seen in vacuoles in the pigment cell columns.

Internalisation of Membrane by Pinocytosis

Pinocytosis of rhabdomere membrane at the bases of the microvilli occurs as a minor mechanism of internalisation. Coated vesicles can be seen still attached to microvilli, and free in the cytoplasm, but they are not frequent, and are at no time seen in sufficient quantities to account for mvbs of the sizes observed.

Involvement of Fixation Artefacts

One component of the shedding profiles illustrated in this paper is probably of artefactual origin: the dense vesicles or "micro-whorls" shown in Figs. 5 and 6 will be shown to be similar to artefacts produced in rhabdomeres of another dipteran,

Lucilia, by prolonged primary fixation or when calcium concentrations no greater than those in the present fixatives are used (Williams, 1980). Other features are independent of fixation method.

Discussion

The present results confirm the reality of extracellular shedding of photoreceptor membrane in the compound eyes of arthropods. In the jumping spider, *Plexippus*, it appears to be a relatively slow event. *Ptilogyna* sheds in bulk, with a dawn peak, and the amount of membrane lost appears to be of similar order to the quantities shed by the spider *Dinopis* (Blest, 1978), and the crabs *Grapsus* (Nässel and Waterman, 1979) and *Leptograpsus* (Stowe, in preparation), although it is not as great. Both *Dinopis* and grapsid crabs show intracellular shedding only. Yamamoto and Yoshida (1978) demonstrate extracellular shedding and phagocytosis of microvilli by the photoreceptors of the simple eye of a holothurian.

Extracellular shedding has not been suspected in insects, despite a wealth of comparative studies of the ultrastructure of compound eyes. It seems likely that it may occur wherever ommatidia contain open rhabdoms whose rhabdomeres abut on extracellular space. Published electron micrographs are uninformative. Schneider and Langer (1969), have presented interesting micrographs of the receptors of the Hemipteran *Gerris*; transverse sections reveal filopodia from the receptors projecting into the intra-ommatidial ECS which they share. One micrograph has large, irregular vacuoles in retinulae. Others disclose conventional mvbs and pinocytotic vesicles. The eyes of this group of Hemiptera require re-examination in relation to the daily cycle, as do those of any arthropod possessing rhabdomeres with non-uniform microvilli.

Trujillo-Cenóz and Bernard (1972) describe the receptors of compound eyes of the dolichopodid fly, *Sympycnus*. The microvilli have a sharply-delimited, distal, tapered and disordered region in most of the receptors shown in their micrographs. It resembles the shedding zone in *Ptilogyna*, and probably has the same functional significance. Surprisingly, microvillar tips of the Megalopteran *Archicauloides* also have a well-defined pale zone, although the rhabdomeres to which they belong form a fused rhabdom, so that the microvillar tips of different retinulae are contiguous (Walcott and Horridge, 1971). Thus, there would seem to be no space available for extracellular shedding. The eye has not yet been examined at the day/night transitions, so that the significance of this pattern of organisation is equivocal.

The control of extracellular shedding is of interest. Transformation of tips of the microvilli occurs well before dawn, so that the sequence leading up to shedding is clearly not a result of simple damage to transductive membrane consequent upon performance. It may be a developmental change that anticipates shedding and allows it to be initiated at the appropriate time. The nature of this change, and the mechanism that defines the zone of transformation so clearly, are not understood. In well-fixed material, transverse sections of microvilli show axial filaments, while adjacent sections of transformed tips do not (Fig. 7). This suggests that shedding may involve or even be controlled by cytoskeletal changes affecting the stability of the microvillar conformation. In the dragonfly, *Hemicordulia*, Laughlin and

McGinness (1978) find that rhabdomeres of the dorsal retina fixed in the light-adapted state show proximal zones where the basal segments of the microvilli are of lower electron-density and are dilated or slightly deformed. This region may prove to be a shedding zone occupying the bases of the microvilli, but it should be remembered that the rhabdoms of crabs, which shed membrane in bulk at dawn (Nässel and Waterman, 1979; Blest et al., 1980), are not regionally differentiated in this way, although those of *Dinopis* may be (Blest, 1978).

Why has the extracellular route been evolved by jumping spiders and crane-flies? For the jumping spider, Blest and Maples (1979) suggested that for shedding at the bases of the microvilli to be possible, the twin rhabdomeres of each cell would have to be separated, and that this geometry would not be compatible with the sharp angular sensitivity functions of the receptors (Hardie and Duelli, 1978) and the high spatial resolution of the eyes. These arguments cannot apply to the tipulid eye, which is designed primarily as a nocturnal eye with low resolution, and has a quite different optical geometry (Williams, in preparation).

Extracellular shedding in both *Ptilogyna* and *Plexippus* offers analogies to vertebrate photoreceptor membrane shedding, although it should be noted that the construction of vertebrate photoreceptors does not allow them to internalise transductive membrane (Young, 1978; Anderson et al., 1978). At present, little is known concerning the addition of membrane material to rhabdomeral microvilli. Attachment of preassembled membrane to the bases of microvilli has been proposed (Itaya, 1976), while autoradiography suggests that membrane proteins or their components can be inserted anywhere in the rhabdomere (Perrelet, 1972; Hafner and Bok, 1977). Such diverse strategies of membrane addition could have very different consequences when membrane is shed. It is clear, however, that distal shedding could allow membrane addition to proceed exclusively at the bases of the microvilli, so that all components would be shed as a function of the time they had been in the rhabdomeres. This hypothesis seems to allow the most economical strategy for turnover, and is suggested to underlie the evolution of extracellular shedding of the kind described in this paper. It rests, however, on the assumption that the high cholesterol content of microvillar membranes reduces their fluidity (Zinkler, 1974), so that translational diffusion of membrane proteins is severely limited. This prediction has been confirmed for crayfish photoreceptors by Goldsmith and Wehner (1977) and Wehner and Goldsmith (1975), but has yet to be extended to other arthropods.

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CHAPTER VI

RHABDOM SIZE AND PHOTORECEPTOR MEMBRANE TURNOVER
IN A MUSCOID FLY

David S. Williams

Summary. The cross-sectional area of rhabdomeres in the compound eye of the blowfly, Lucilia, was found to remain constant under 12 h light/12 h dark cyclic lighting, and 10 days constant light or darkness. It was reduced slightly during 3 h light after 10 days darkness (by 21%), or on exposure to 2 h darkness + 1½ h light after 10 days light (by 10%). Morphological evidence indicates that shedding of photoreceptor membrane during turnover is achieved by an extracellular route, and by pinocytosis from the bases of the microvilli. The photoreceptor membrane shed by both mechanisms appears to accumulate in multivesicular bodies. The amount of photoreceptor membrane shedding, as indicated by numbers of multivesicular bodies, is constant throughout the day and night on cyclic lighting, decreases in constant darkness, but returns to its normal level after an exposure to 3 h light subsequent to 10 days darkness.

Key words: Fly - Rhabdom size - Photoreceptors - Microvilli - Membrane turnover.

Turnover of photoreceptor membrane appears to be fundamental to the function of all photoreceptors (Young 1976; Blest 1980). As a result of a large proportion of phototransductive membrane turning over each day, and the separation of the shedding and assembly phases of turnover, the rhabdoms of many arthropods vary in size on a daily cycle. Generally, shedding peaks at dawn, while assembly of new membrane occurs at dusk (Blest 1978; Stowe

1980; Williams 1982) or is continuous (White et al. 1980). Accordingly, these variable-size rhabdoms are smaller during the day than at night.

The compound eyes of advanced dipterans have been employed in an extensive range of physiological, behavioural, optical, and theoretical investigations, the results of which have often been dependent on the cross-sectional area of the distal ends of the rhabdomeres. Changes in cross-sectional area could affect the waveguide characteristics of a rhabdomere (Kirschfeld and Snyder 1975). Optical properties that might be affected as a result include angular and absolute sensitivities (Pask and Snyder 1975), spectral sensitivity (Snyder and Miller 1972; Snyder and Pask 1973), and inter-rhabdomeral crosstalk (Wijngaard and Stavenga 1975). Therefore the question of whether or not rhabdom size in advanced flies varies is pertinent to our understanding of dipteran vision. From a preliminary attempt to establish the mechanisms of photoreceptor membrane shedding during turnover in an open rhabdom (Williams and Blest 1980), it was discovered that in muscoid flies there was no daily peak of shedding that could be determined from obvious changes in rhabdomeral ultrastructure or size. The present paper examines in more detail rhabdom size and the shedding of photoreceptor membrane during turnover in the compound eye of the blowfly, Lucilia, under a daily light cycle and abnormal light regimes.

Materials and Methods

Animals

Laboratory-bred pupae of the sheep blowfly, Lucilia cuprina, emerged in a wire-mesh cage supplied with water

and raw sugar ad lib. Daylight fluorescent lighting (2 x 40 W tubes 40-50 cms from the cage; illuminance in the centre of the cage was 1000 lux) was provided from 0600 to 1800 h daily, and temperature was $23 \pm 4^\circ \text{C}$. Groups of about 20 flies up to five days old were placed in one of five cages for 10 days. One cage remained under the 12 h light/12 h dark cyclic lighting. Eyes from flies in this cage were fixed at 0900 h, 1200 h, 1500 h, 2100 h, 2400 h, 0300 h on Day 11. The other cages were exposed to constant light, constant light followed by 2 h darkness then $\frac{1}{2}$ h light, constant darkness, or constant darkness followed by 3 h light. Light in the centre of each cage was 1000 lux; the cages were surrounded by white cardboard with daylight-fluorescent lighting above. Darkness was complete. Temperature was $23 \pm 2^\circ \text{C}$, humidity $48 \pm 2\%$, and water and raw sugar were provided ad lib. Animals subject to these experimental light regimes were introduced to their new light regime at 0600 h, and their eyes were fixed between 1100 h and 1200 h on the eleventh day. The particular regimes were chosen because regimes of this type had been shown in vertebrates to affect shedding and induce changes (e.g., Besharse et al. 1977; Currie et al. 1978; Battelle and LaVail 1978) in an otherwise stable rod outer segment size (Basinger et al. 1976). Ten days constant light as stated in the text, is in fact 10 days + 2 h constant light, and 10 days constant darkness actually 10 days + 14 h constant darkness.

Tissue Preparation

Light-adapted flies were dissected under light from a tungsten microscope lamp filtered by a Schott KG3 heat filter. For dark-adapted flies, an additional Schott RG 660 red cut-off filter were placed in front of the lamp.

The heads of the flies were cut open with microscissors while immersed in primary fixative: 2.5 % glutaraldehyde + 2 % paraformaldehyde buffered in 0.1 M sodium dihydrogen orthophosphate-NaOH with 0.07 M D-glucose + 2 mM EGTA. The last obviates the artefactual effects of free Ca^{++} during fixation (Williams 1980a; White and Michaud 1981). The tissue around one compound eye was cut away, and the dorsal extremity of that eye cut open. Thus one eye from each fly was quickly exposed to the fixative without distorting it.

After several hours in the fixative, the central region of the eye was cut out with a razor blade. This block of retina was then briefly washed in buffer, post-fixed in 1% OsO_4 in the same phosphate buffer, dehydrated through an ethanol series, and embedded in Araldite. Silver-grey sections were cut with glass knives, collected on formvar-coated slot grids, stained with saturated aqueous uranyl acetate followed by Reynolds' lead citrate, and examined in an Hitachi H500 or H600 electron microscope.

Measurement of Rhabdomeral Cross-sectional Area

Because the interommatidial angle in the centre of the eye is no more than 3° (Stavenga, 1975), about 20 ommatidia covering a circular area in the centre of a given transverse section of a retina could be considered to be in "true" transverse section. Transverse sections for electron microscopy were taken of these ommatidia at the level of the nuclei of R1-6: 20-30 μm from the distal ends of the rhabdomeres. For a given rhabdomere, cross-sectional area is fairly constant over this length (Boschek 1971). The sections were magnified 10,000 times and the cross-sectional areas of the rhabdomeres of R3 and

R7 were measured from micrographs using a Kontron MOP-AM03 image analyser.

Counting of Multivesicular Bodies

Transverse sections of R1-7 taken just distal or proximal to the nuclear layer of R1-6 were scanned in the electron microscope at a magnification of 20,000 times. A x10 binocular scope was swung into position to verify the sighting of an multivesicular body. Multivesicular bodies were counted from 30-40 ommatidia per eye. Counts were made from six eyes after 10 days + 3 h darkness, six eyes after 10 days darkness + 3 h light, four eyes at both 1200 h and 2400 h on cyclic lighting, and two eyes at each of the following times on cyclic lighting: 0900 h, 1500 h, 2100 h, and 0300 h.

Results

Cross-sectional Area of Rhabdomeres

The retinae of the compound eyes of muscoid flies consist of an array of open rhabdoms (Dietrich 1909; Trujillo-Cer6z and Melamed 1966; Boschek 1971). The cross-sectional area of the rhabdomeres, 20-30 μm from their distal extremities, is presented in relation to different light regimes in Table 1. The rhabdomere of R3 was taken as representative of the outer rhabdomeres, R7 of the central rhabdomere. No significant difference was found between the cross-sectional area of rhabdomeres at midday and midnight on cyclic lighting, indicating that the width of the rhabdomeres is probably constant on a normal daily light cycle. Furthermore, the cross-sectional area did not change significantly from that measured under cyclic lighting at 1200 h after constant

Table 1. Cross-sectional area of rhabdomeres

Light regimes ¹	Number of eyes	Mean area (μm^2) + 1 S.E.	Probability of no difference ²
<u>A</u> LD (fixed: 1200h)	4	R3 0.701 \pm 0.043 R7 0.317 \pm 0.009	
<u>B</u> LD (fixed: 2400h)	4	R3 0.683 \pm 0.054 R7 0.319 \pm 0.028	cf. <u>A</u> : n.s.d. n.s.d.
<u>C</u> LL \rightarrow 3 1/2 h L	8	R3 0.748 \pm 0.032 R7 0.354 \pm 0.0125	cf. <u>A</u> : n.s.d. n.s.d.
<u>D</u> LL \rightarrow 2h D + 1 1/2 hL	10	R3 0.670 \pm 0.025 R7 0.346 \pm 0.012	cf. <u>C</u> : p < 0.05 n.s.d.
<u>E</u> DD \rightarrow 3h D	6	R3 0.669 \pm 0.014 R7 0.289 \pm 0.010	cf. <u>A</u> : n.s.d. n.s.d.
<u>F</u> DD \rightarrow 3h L	6	R3 0.528 \pm 0.037 R7 0.258 \pm 0.029	cf. <u>E</u> : p < 0.01 n.s.d.

¹ LD, cyclic lighting; LL 10 days constant light; DD 10 days constant darkness.

² n.s.d., no significant difference at 5% level (t-test). Each comparison is made with the appropriate control.

light or constant darkness.

The cross-sectional area of the R3 rhabdomere was found to be slightly but significantly smaller than that of the controls in: (1) eyes exposed to 10 days light, followed by 2 h dark and 1½ h light (10% smaller; $p < 0.05$), and (2) eyes exposed to 10 days darkness, followed by 3 h light (21% smaller; $p < 0.01$). The controls were exposed to an equivalent time of uninterrupted light or darkness. No significant difference was found for the R7 rhabdomere.

Cross-sectional area of the rhabdomeres can therefore be considered to remain constant, except under extremely artificial lighting conditions, and even then the change is very small.

Photoreceptor Membrane Shedding

Examinations of retinæ of Lucilia fixed at regular intervals over a daily cycle did not reveal a pronounced peak of photoreceptor membrane shedding. Nevertheless, profiles were observed that suggest the existence of two routes of membrane shedding.

Evidence for Extracellular Shedding. Microvilli at the sides of the rhabdomeres often had a less regular configuration than those adjacent to them, and were often detached from their retinular cell (Figs. 1a, b, 2a, b). Similarly, short distal segments of the microvilli occasionally appeared deformed (e.g., Fig. 3a). In the extracellular space surrounding the rhabdomeres, lightly-stained tubular segments were frequently apparent (Fig. 1b, c). Occasionally, this membranous detritus in the extracellular space appeared to be undergoing phagocytosis by the retinular cells (Fig. 2a-c). Vacuoles containing partially vesiculated membrane (Fig. 2d, e),

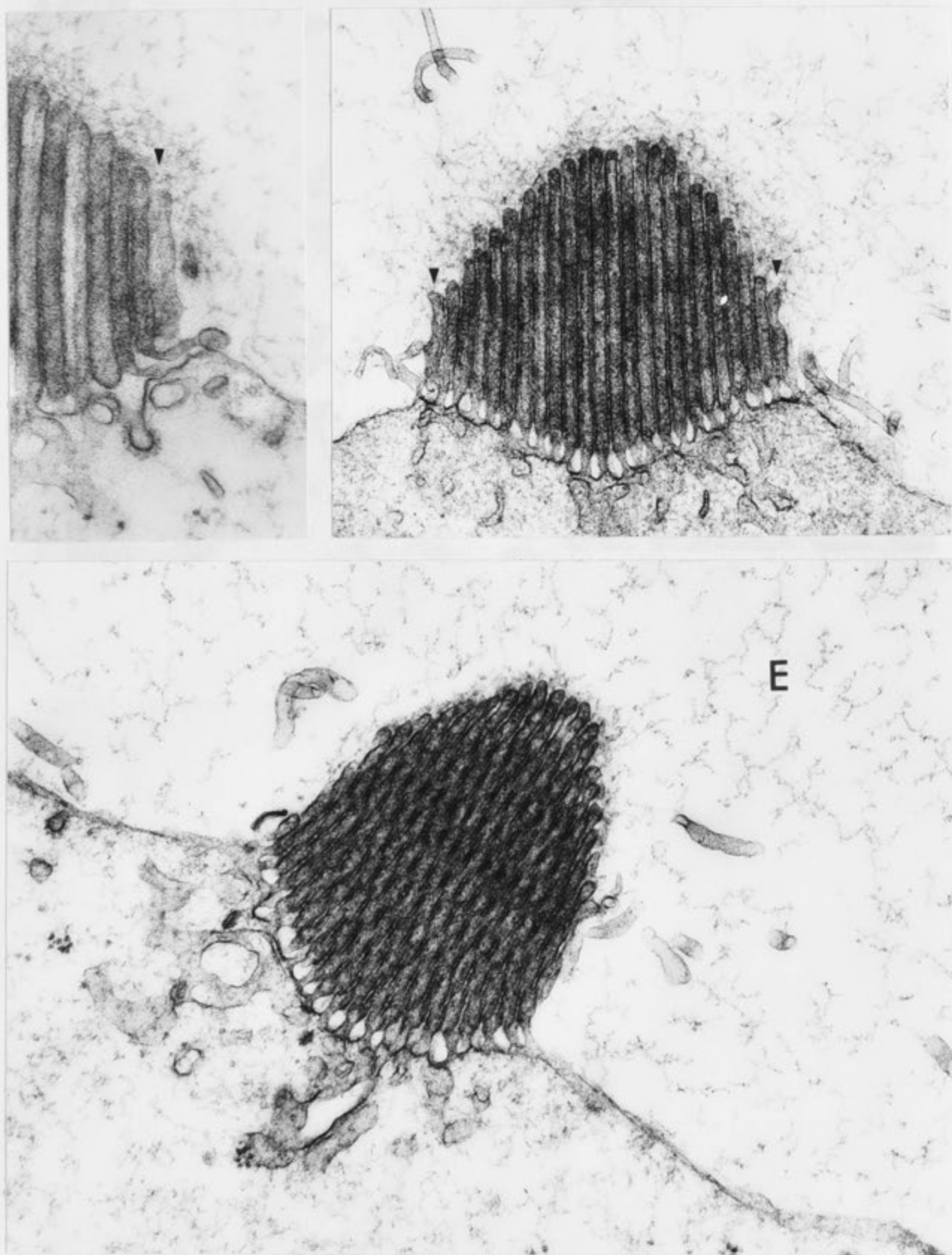


Fig. 1. a, b (top) Microvilli at the margins of rhabdomeres have less regular profiles (arrows). Some have become detached from their reticular cell. c (bottom) Lightly-stained segments of detached microvilli are apparent in the surrounding extracellular space (E). a, X 70,000; b X 44,000; c, X 50,000.

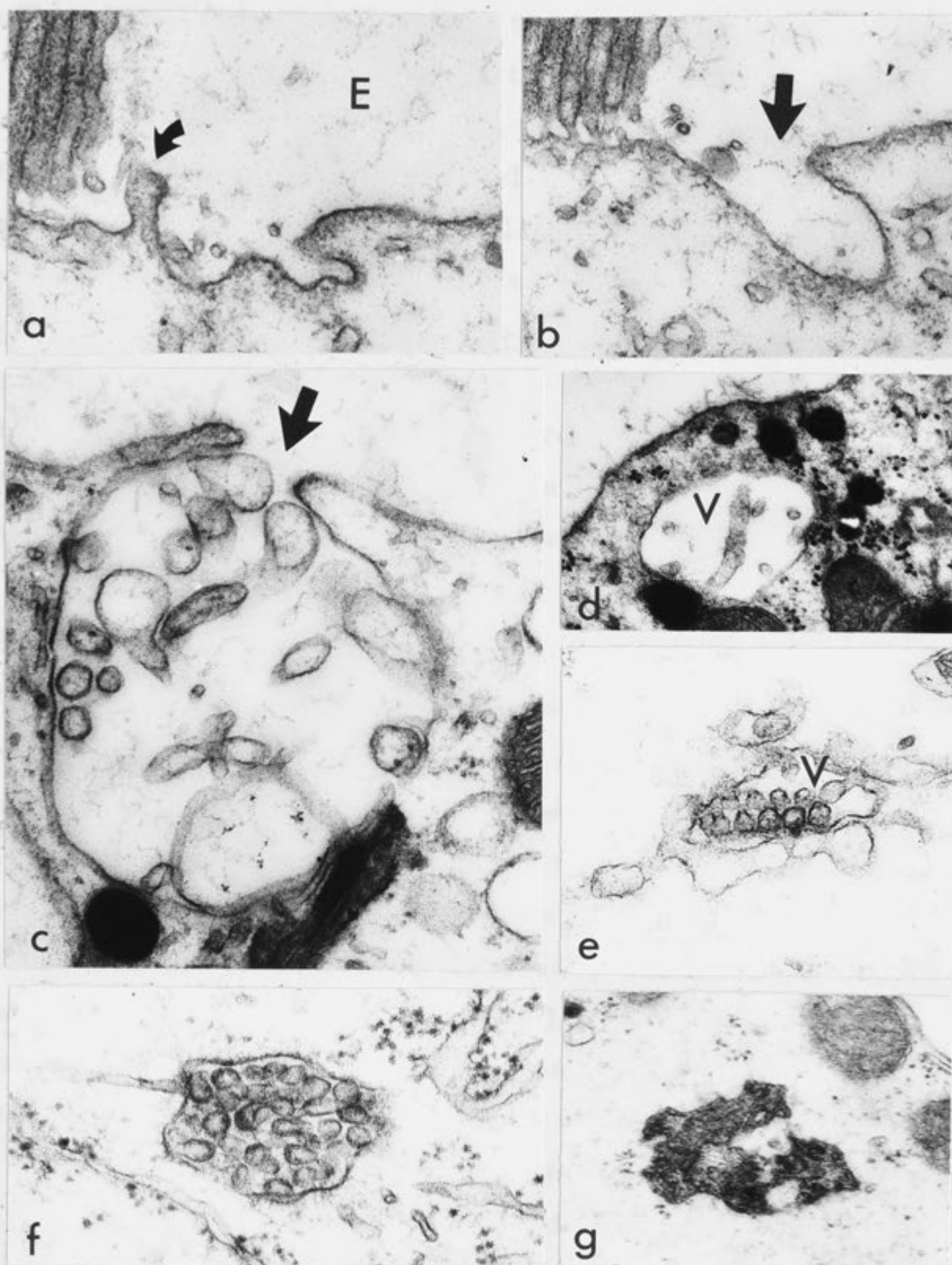


Fig. 2. a-c Profiles of phagocytosis of membranous detritus from extracellular space (E). Curved arrow (a), indicates pseudopodium. Large arrows (b, c) indicate continuity between phagocytotic vacuole and extracellular space. d, e Vacuoles (V) within reticular cells, containing partially vesiculated membrane, considered to be derived from shed microvilli. f Multivesicular body. g Residual body. a X 68,000; b X 56,000; c X 60,000; d X 43,000; e X 46,000; f X 56,000; g X 35,000.

multivesicular bodies (mvbs) (Fig. 2f), and residual bodies (Fig. 2g) were found in the retinular cells.

These observations suggest that microvillar membrane is shed from the sides of the rhabdomeres and distal ends of the microvilli into extracellular space, from where it is phagocytosed by the retinular cells, and subsequently degraded through mvbs to other secondary lysosomes.

Evidence for Shedding by Pinocytosis. Coated pits and vesicles were found around the bases of the microvilli (Fig. 3a) and further into the retinular cells near mvbs (Fig. 3b), so that some photoreceptor membrane appears to shed by endocytosis, followed by accumulation in mvbs.

Multivesicular Bodies

The above observations suggest that both the proposed routes of photoreceptor membrane disposal during turnover include the aggregation of shed membrane in multivesicular bodies. Accordingly, provided that there is no other significant source of the multivesicular bodies, and that their rate of breakdown is constant, the rate of shedding under different conditions should be reflected by the number of these secondary lysosomes in the retinular cells.

Numbers of multivesicular bodies were rather low in all cases (Fig. 4), but a significant difference ($p < 0.05$) was found between 10 days + 3 h darkness and 10 days darkness + 3 h light; they were almost twice as abundant in the latter (Fig. 4). A significant difference in their number was also found after 10 days + 3 h darkness compared to 1200 h on cyclic lighting ($p < 0.05$). No significant difference was found between midday and midnight on cyclic lighting (Fig. 4). At each of the other times sampled on the daily cycle, an average of 1.1

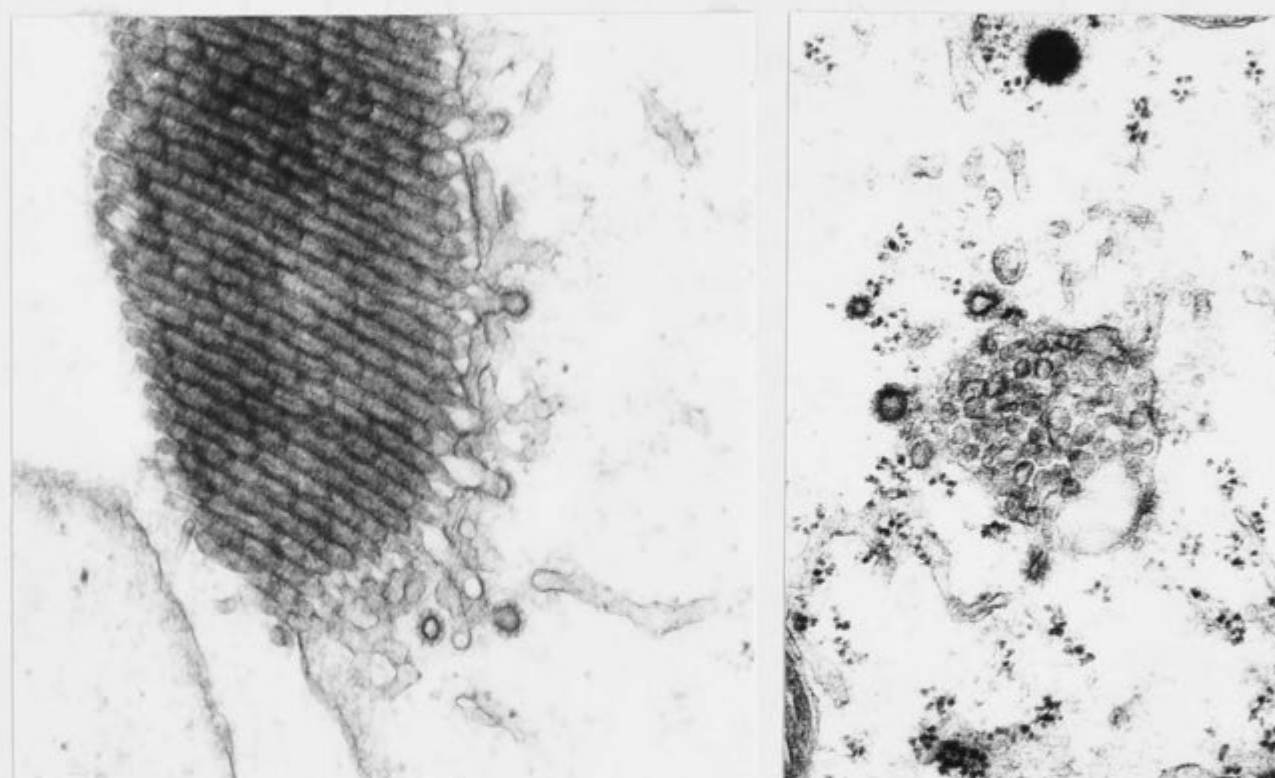


Fig. 3. a (left) Coated pits and vesicles in an unusually large number, at the bases of microvilli. The distal segments of the microvilli are somewhat deformed. X 33,000. b (right) Coated vesicles near a multivesicular body in a reticular cell. X 39,000.

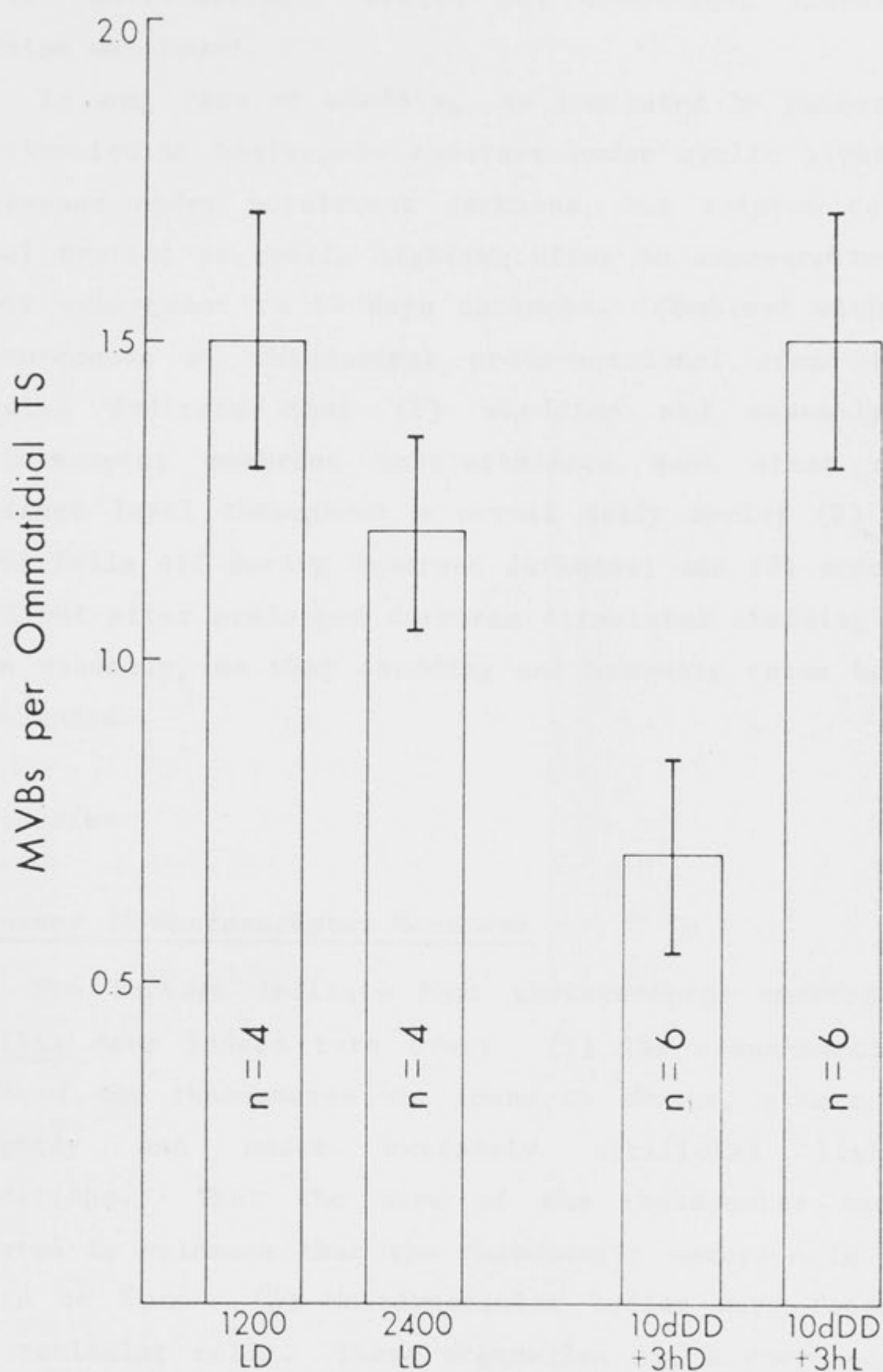


Fig 4. Histogram of mean numbers of multivesicular bodies counted per ommatidium in transverse section at midday and midnight on daily cyclic lighting (LD), and after 10 days + 3 hours darkness (DD), and 10 days darkness followed by 3 hours light. Error bars indicate 1 standard error of the mean.

- 1.7 multivesicular bodies per ommatidial transverse section was found.

In sum, rate of shedding, as indicated by numbers of multivesicular bodies, is constant under cyclic lighting, decreases under persistent darkness, but returns to the level typical of cyclic lighting after an exposure to 3 h light subsequent to 10 days darkness. Combined with the measurements of rhabdomeral cross-sectional areas these results indicate that (1) shedding and assembly of photoreceptor membrane counterbalance each other at a constant level throughout a normal daily cycle; (2) this level falls off during constant darkness; and (3) exposure to light after prolonged darkness stimulates shedding more than assembly, so that shedding and assembly rates become imbalanced.

Discussion

Turnover of Photoreceptor Membrane

Two factors indicate that photoreceptor membrane in Lucilia does indeed turn over: (1) The cross-sectional area of the rhabdomeres was found to change, albeit only slightly and under extremely artificial lighting conditions. That the size of the rhabdomeres can be altered is evidence that the rhabdomeric membrane is in a state of flux. (2) Multivesicular bodies were found in the reticular cells. These organelles are a component of the shedding pathway during photoreceptor membrane turnover in nearly all arthropods, irrespective of the route employed (Eguchi and Waterman 1967, 1976; White 1968; Blest 1978; Williams and Blest 1980).

Given that photoreceptor membrane does turn over in Lucilia, one question is how? Morphological evidence has

been presented above for two mechanisms of shedding during turnover. The existence of these mechanisms is supported by analogy with a more primitive dipteran in which similar methods of shedding are convincingly evident on a daily cycle.

In the tipulid, Ptilogyna, photoreceptor membrane that is about to be shed (from the distal ends of the microvilli into extracellular space) is less strictly aligned and stains more lightly than the rest of the rhabdomere after conventional fixation and staining (Williams and Blest 1980), due to local deletion of its cytoskeletal axial filament (Blest et al. 1982). Additionally, it is more susceptible to the formation of myelin-like artefactual whorls when fixed in buffers containing Ca^{++} (Williams 1980a). In Lucilia, complete microvilli at the sides of the rhabdomeres, and distal segments of the inner microvilli were observed without a strict configuration, lightly stained, and sometimes detached (Fig. 1). Moreover, membrane from these marginal parts of the rhabdomeres collapse more readily into myelin-like whorls when fixed in the presence of Ca^{++} than that of the rest of the rhabdomere (Williams 1980a). White and Michaud (1981) also found an association between the formation of similar whorls and photoreceptor membrane about to be shed in another dipteran, a mosquito. Analogous structure and tendency to form artefacts therefore indicates that during normal turnover in Lucilia microvillar membrane is probably shed from the margins of the rhabdomeres into extracellular space.

In Ptilogyna, the membranous detritus that results from shedding is phagocytosed from the extracellular space by the retinular cells, where it accumulates as vesicles within multivesicular bodies, and is then degraded to

residual bodies (Williams and Blest 1980). In Lucilia, the detached microvillar membrane appears to meet the same fate (Fig. 2); it has also been observed undergoing phagocytosis in its artefactual myelin-like whorl state (Williams 1980a).

Another possible means of extracellular shedding was suggested by Blest and Maples (1979) by analogy with the anterior-lateral eye of a jumping spider, in which the tips of the photoreceptor microvilli disintegrate into amorphous material in the extracellular space. Blest and Maples were prompted to make their proposal from the presence of clouds of an electron-opaque substance around the tips of the microvilli in previously published electron micrographs. However, this substance (e.g., Fig. 1) is continuous with the rhabdomere "caps" (Trujillo-Cenóz and Melamed 1966; Schneider and Langer 1966), and appears to be identical with the material of which they are composed. It could alternatively be a surface coat of the microvilli, analogous to the mucopolysaccharide coat found on the surface of mammalian gut microvilli (Ito 1965). Like the surface coat of gut microvilli (Ito 1965), the rhabdomeres of the housefly, Musca, give a positive Periodic acid-Schiff reaction (Suzuki 1962).

The common means of photoreceptor membrane shedding in arthropods that have rhabdomeres unexposed to a large extracellular space is by pinocytosis from the bases of the microvilli, followed by the aggregation of the vesicles into multivesicular bodies (Eguchi and Waterman 1967, 1976; White 1968; Blest 1978; Martin and Hafner 1981). In the open rhabdom of Ptilogyna, this method occurs to a secondary extent (Williams and Blest 1980). It also appears to occur in Lucilia.

Cross-sectional Area of Rhabdomeres

The cross-sectional area of the rhabdomeres in Lucilia remained fairly constant even after extremely artificial lighting regimes. In contrast, many arthropods have rhabdoms that vary in size by several-fold on a daily cycle (e.g., Blest 1978; Nassel and Waterman 1979; Stowe 1981; Williams 1980b, 1982). The current finding has particular relevance to the large amount of previous work on the photoreceptor function of advanced dipterans. Invariably, these investigations have been made without consideration of the time of day or the lighting regime to which the experimental animals were exposed. The present results show, however, that the results of these investigations can generally be considered applicable under most circumstances, including the natural conditions of the fly.

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CHAPTER VII

THE HISTORY OF THE
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OMMATIDIAL STRUCTURE IN RELATION TO
PHOTORECEPTOR MEMBRANE TURNOVER IN THE LOCUST

David S. Williams

Summary. In the compound eye of the locust, *Locusta*, the cross-sectional area of the rhabdoms increases at "dusk" by 4.7-fold due to the rapid assembly of new microvillar membrane, and decreases at "dawn" by a corresponding amount as a result of pinocytotic shedding from the microvilli. The rhabdoms at night have more and longer photoreceptor microvilli than rhabdoms during the day. The orientations of the six rhabdomeres that comprise the distal rhabdom also change. The density of intramembrane particles on the P-face of the microvillar membrane, putatively representing mostly rhodopsin molecules, or aggregates thereof, does not change.

An alteration in the size of the ommatidial field-stop, produced by the primary pigment cells, is concomitant with the change in rhabdom size. At night the increase in size of the field-stop must widen the angular acceptance of a rhabdom, increasing the capture of photons from an extended field. Conversely, during the day, when photons are more abundant, its decrease must narrow the acceptance angle, increasing angular resolution. Because of the presence of this field stop, the optics of the ommatidium would not be greatly affected if the rhabdom were to remain always at its night size. It is argued, therefore, that the variable-size rhabdom must have resulted from some demand other than that of light/dark adaptation.

Changes in size and organisation of the rhabdoms in response to various light regimes indicate that: (1) Rapid

shedding of photoreceptor membrane is induced by the onset of light, but shedding also occurs slowly in darkness during the day. (2) Microvillar assembly is initiated by the onset of darkness, but also occurs at the normal time of dusk without a change in ambient lighting, provided there has been some light during the day. Therefore, both shedding and assembly of microvillar membrane are affected by the state of illumination, but also appear to be under some endogenous control.

Key words: Grasshopper - Photoreceptors - Microvilli - Membrane turnover - Freeze fracturing - Biological clock.

Following the original electron microscopical studies of the effects of light and darkness on rhabdom size in the larval mosquito eye by White (1967), it has been established that the rhabdoms of many invertebrates decrease in width (and in some cases length also) at the onset of light (normally dawn), and increase at the onset of darkness (normally dusk). "Variable-size rhabdoms" of this kind have been found in: (1) Invertebrates exclusive of arthropods: holothurian ocelli (Yamamoto and Yoshida 1978), seastar ocelli (Eakin and Brandenburger 1979), cephalopod molluscs (Young 1962); (2) Crustaceans: shrimps (Debaisieux 1944; Itaya 1976; Hertel 1980), crabs (Nassel and Waterman 1979; Stowe 1980a); (3) Chelicerates: spiders (Blest 1978; Blest and Day 1978; Blest, et al. 1980), harvestmen (Schliwa 1979), Limulus lateral eye (Behrens 1974; Miller and Cawthon 1974); (4) Insects: larval mosquito ocelli (White and Lord 1975), adult mosquito compound eye (Sato et al. 1957; Brammer and Clarin 1976; Brammer et al. 1978), mycetophilid larval (glowworm) ocelli and adult compound eye (Meyer-Rochow and Waldvogel 1979), adult tipulid (Williams 1980a), mantid and locust

(Horridge et al. 1981). The change in rhabdom size is achieved by the daily turnover of large amounts of photoreceptor membrane and a separation of the peaks of the shedding and assembly phases of the turnover (Blest 1980); the Limulus lateral eye possibly provides an exception (Chamberlain and Barlow 1979; but see also discussion by Stowe 1981).

Notwithstanding reports that locusts have variable-size rhabdoms (Horridge and Blest 1980; Horridge et al. 1981), the daily changes in rhabdom structure have not been described in detail. The present paper examines the rhabdom structure and associated optics of the ommatidium in Locusta, in relation to time of day and state of illumination. The photoreceptor cells of locusts are amenable to intracellular recording for long periods of time, and are therefore highly suitable for investigating the functional effects of photoreceptor membrane turnover (Horridge et al. 1981). The following description provides essential background for such studies. In addition, it gives insight into the control of photoreceptor membrane turnover.

Materials and Methods

Adult Locusta migratoria L. and Valanga irregularis (Walk.) were taken from a laboratory culture that was fed bran and wheat and maintained on a 12 h light: 12 h dark cycle (lights on, i.e. "dawn", at 0600 h; "dusk" at 1800 h) at 25-35°C, for at least two weeks prior to experimentation. Light was provided by a 60W incandescent bulb in each cage (36 x 36 x 46 cm), and two 40W daylight-fluorescent lights shared by three cages (illuminance in the centre of the cage was 1500 lux). At night, lights

were off and no light entered through any windows.

Compound eyes of Locusta were fixed at particular times on the normal light regime and on five experimental regimes (as shown in Fig. 8). During at least 12 h immediately prior to fixation, animals were kept at $25 \pm 1^\circ\text{C}$ and $48 \pm 1\%$ relative humidity, and under the following illumination: for light adaptation, under daylight fluorescent lights of similar intensity to that used during the day previously; for dark adaptation, the only lighting was a Kodak 1A safelight (15W bulb). The eyes were dissected under the appropriate illumination (fluorescent lights or the safelight). Dissections were made so as to leave the central region of the eye (Fig. 1) intact and undistorted. The head was cut in half, one half was discarded, then a fresh razor blade was used to slice through the surface of the remaining eye. The slice was initiated through the cornea at about the midline, and directed ventrally, in such a way that no cut was made through the basement membrane, and all the ommatidia dorsal to the cut were undistorted. The half-head was then quickly immersed in primary fixative: either 2.5% glutaraldehyde + 2% paraformaldehyde buffered in 0.08 M sodium dihydrogen orthophosphate-NaOH plus 0.06 M D-glucose, or 2.5% glutaraldehyde buffered in 0.08 M sodium cacodylate plus 0.11 M sucrose. No calcium was added to the buffer solutions (Williams 1980b). After 3-12 h primary fixation at 4°C , samples were transferred to buffer without fixative, and the dissection was completed so that only a small block of the ommatidia, lying immediately dorsal to the initial slice, remained (Fig. 1). Following this method of fixation, no whorls of membrane, described by Horridge and Barnard (1965) as "onion bodies", were evident in the retinular cells.

For conventional microscopy, tissues were osmicated in the same buffer, dehydrated through an ethanol series and propylene oxide, and embedded in Araldite. Thin sections were collected on formvar-coated slot grids and stained with uranyl acetate followed by Reynolds' lead citrate. They were examined in a Zeiss 109 electron microscope. Measurements of the cross-sectional areas of the rhabdoms were made with a Kontron MOP-AM03 image analyser, from micrographs of rhabdoms in true transverse section (e.g., Fig. 2), magnified 3300 times.

Adult Valanga were used in the freeze-fracture experiments since no Locusta were available at the time. To prepare replicas, glycerol was slowly added to the wash solution, containing pieces of retina that were dissected and fixed as above, to give a concentration of 25-30%. Blocks of retinae were frozen in Freon-22, stored in liquid nitrogen, and fractured at -115° to -105°C and 10^{-6} to 10^{-7} torr in a Balzers BAF 300 freeze-etch unit. Specimens were not etched before platinum/carbon coating, which was controlled with a quartz thin-film monitor. Replicas were cleaned in chromic acid (5% sodium dichromate in 50% sulphuric acid), rinsed in distilled water, collected on formvar-coated slot grids, and examined in an Hitachi H500 microscope. The particle density on the P-face of the microvillar membrane was measured from prints magnified at least 100,000 times, using the Kontron MOP-AM03 image analyser. Particles in only the central 50-nm of the microvillar profile were considered. To compare particle densities between day and night-state eyes about 200 particles were counted from each of 30 rhabdoms sampled from four day-adapted animals and 30 rhabdoms sampled from four night-adapted animals.

To test the significance of differences in rhabdom

size, intramembrane particle density, and microvillar diameter, the two-sample t-test (two tail) was used. Usage of this test was justified on the grounds that the two data samples were unrelated, had similar variances, and were from populations that did not deviate too strongly from a normal distribution (e.g., were not obviously skewed).

Results

Ommatidial Structure and Optics

Each compound eye of an adult Locusta has about 8500 ommatidia packed in an hexagonal array (Shaw 1978). A region of higher acuity, where the interommatidial angle is smaller, accepts light from in front of the animal (Horridge 1978). The present paper examines ommatidia from the centre of the eye (Fig. 1), where the interommatidial angle is fairly constant. The ommatidial organisation has been described by Wilson et al. (1978). Beneath the lenslet, four cone cells taper proximally (Fig. 3) and penetrate about 10 μm into the centre of the rhabdom. The rhabdom is about 300 μm long. Two primary pigment cells surround the cone cells, and secondary pigment cells ensheath the entire ommatidium. Light is brought to focus near the most distal extremity of the rhabdom (McIntyre 1982), and here the primary pigment cells provide the ommatidium with a field stop (Fig. 3b). This field stop is about 1.5 times greater in diameter than the rhabdom at the level of the distal nuclear layer (about 50-100 μm proximal to the field stop), irrespective of the size of the rhabdom. The position of the field stop is the same at night as during the day; it is 80-85 μm from the flat distal end of the

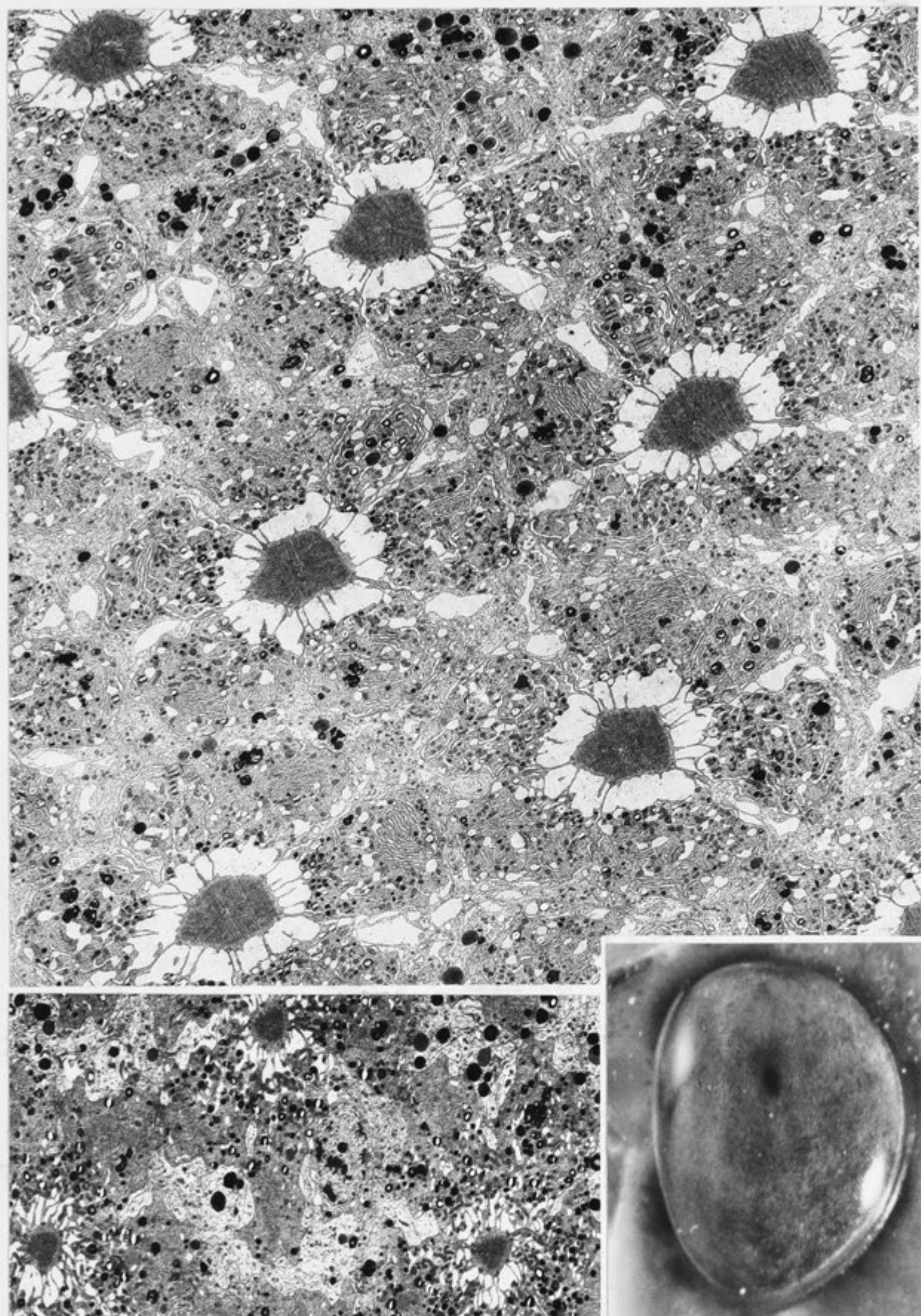


Fig. 1. (lower right) Photograph of left compound eye of Locusta migratoria. The present paper considers ommatidia from the centre of the eye. X 15.

Fig. 2. Transverse sections through the retina in the central region of a compound eye of Locusta. Typical of the sections used for measurements of rhabdom cross-sectional areas. a (top) normal night-state; b (bottom) normal day-state. a and b: X 2300.

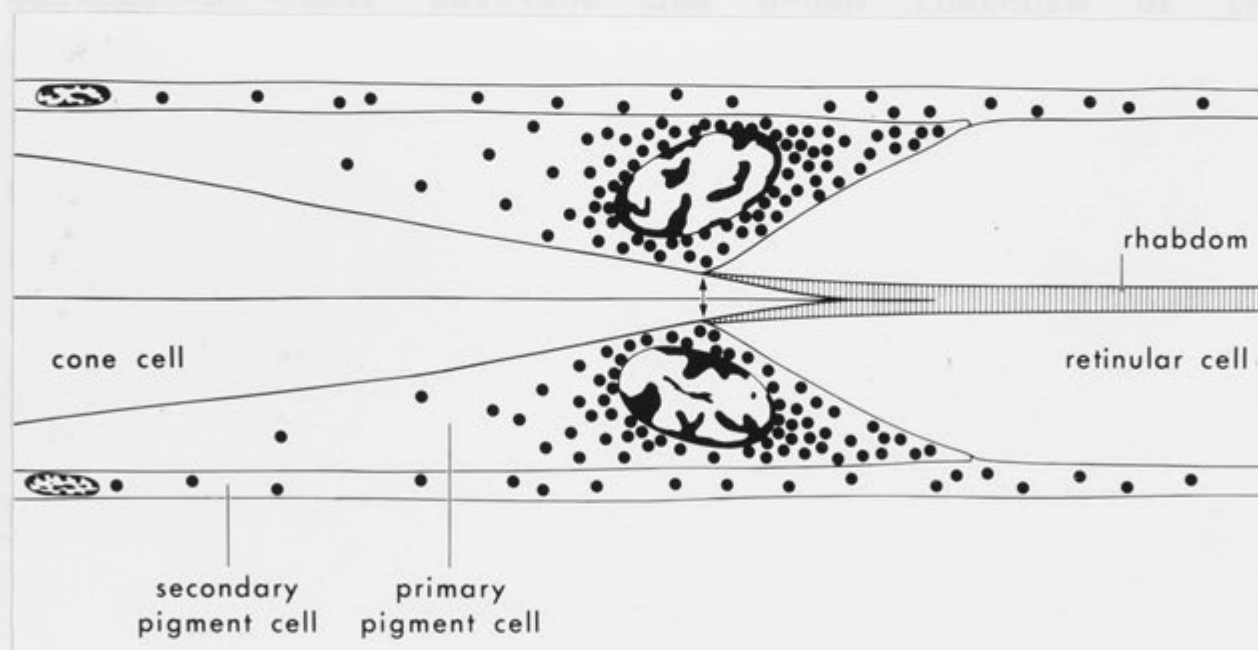
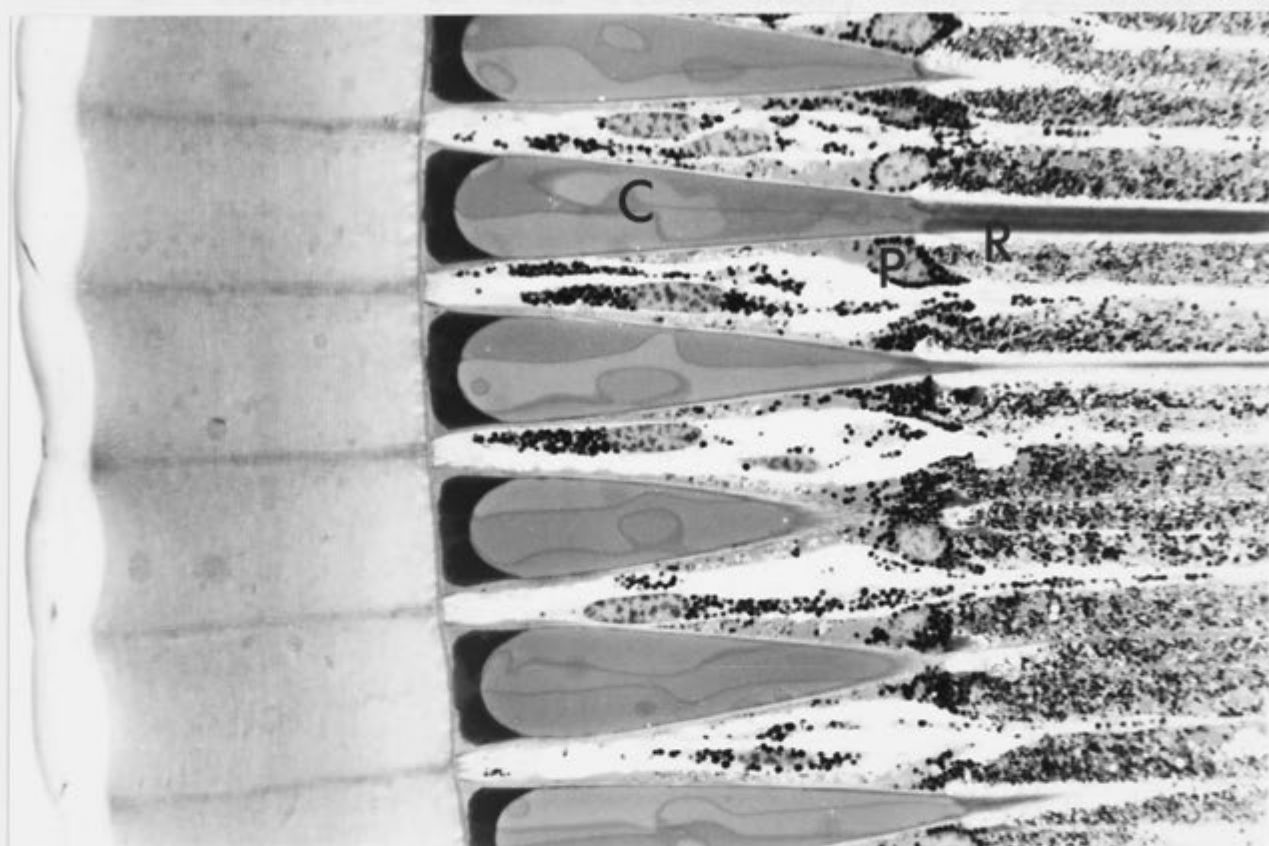


Fig. 3. a (top) Light micrograph of longitudinal section through lenslets, the cone (C), and distal end of the rhabdom (R). The nuclei of the cone cells cap each cone. P, primary pigment cell. X 600. b (bottom) Diagram of base of cone and distal end of rhabdom. The primary pigment cells effect a field-stop, which is indicated by the arrow.

cone cells (Fig. 3a). The secondary pigment cells do not change markedly between light and dark adaptation.

In addition to the field stop, the "palisade" of vacuoles of endoplasmic reticulum, which surrounds the rhabdom when dark-adapted (Horridge and Barnard 1965) (Figs. 2a, 4a), should also affect the amount of light captured by the rhabdom. Some doubt concerning the effectiveness of the palisade has been expressed by Shaw (1978), who found it to persist in light-adapted ommatidia. In the present study, the distal regions of retinae fixed within a few hours after the onset of light were found to have their perirhabdomal palisades replaced by dense cytoplasm containing pigment granules and mitochondria. This condition was not so obvious in eyes that had been light-adapted for some time (e.g., fixed in the afternoon on a normal light cycle). An interplay between a clear palisade and dense contents of the cytoplasm should affect the light absorption of the rhabdom by changing its light-guiding properties (Horridge and Barnard 1965), and is probably important as a short-term means of adaptation when illumination changes (cf. the longitudinal pupil mechanism found in many other insects: review by Stavenga 1979).

Rhabdom Structure on a Daily Cycle

Between night and day the cross-sectional area of the distal rhabdom changes 4.7-fold (Fig. 4, Table 1). The distal part of the rhabdom contains a central cone thread and tapers. Therefore, for reasons of accuracy, measurements of the cross-sectional area of rhabdoms were made proximal to the central cone thread and distal to, or including the distal nuclear layer; i.e. along a length of rhabdom of about 30 μm with an almost uniform diameter.

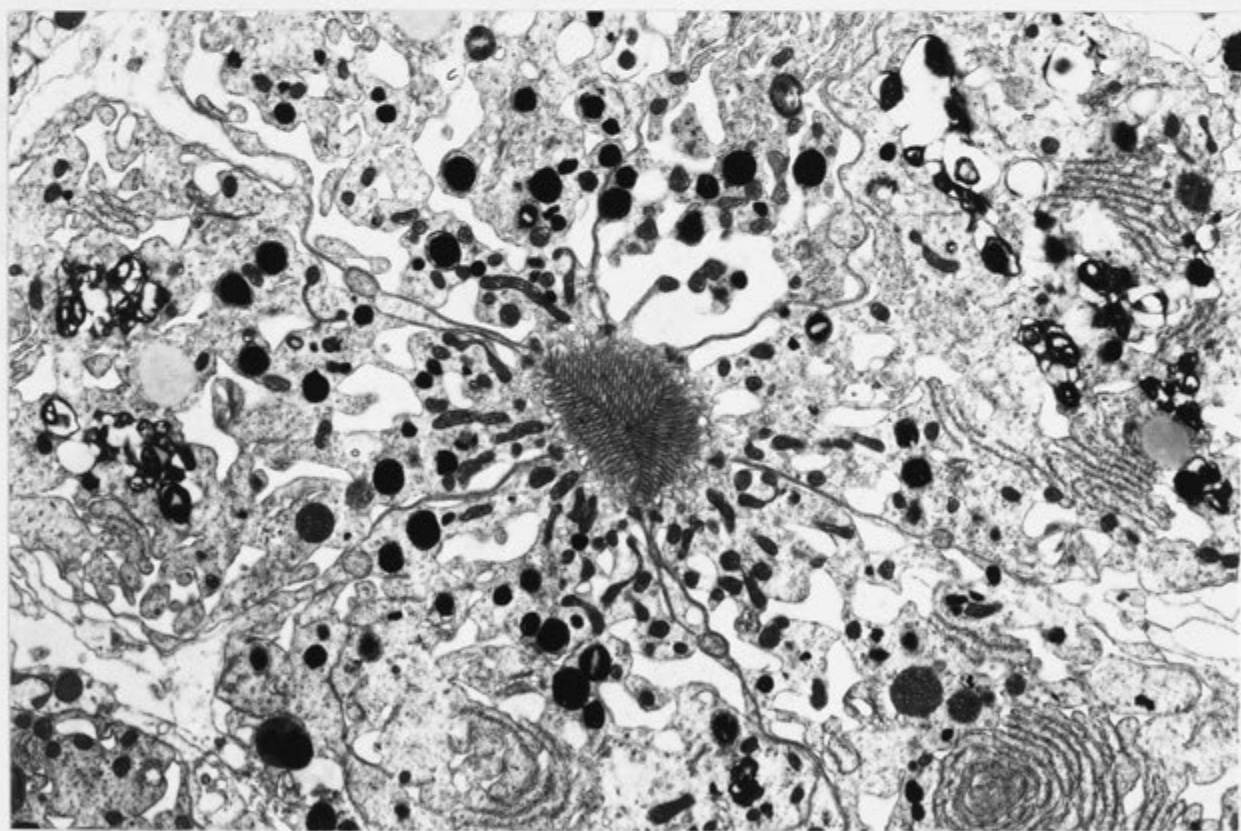
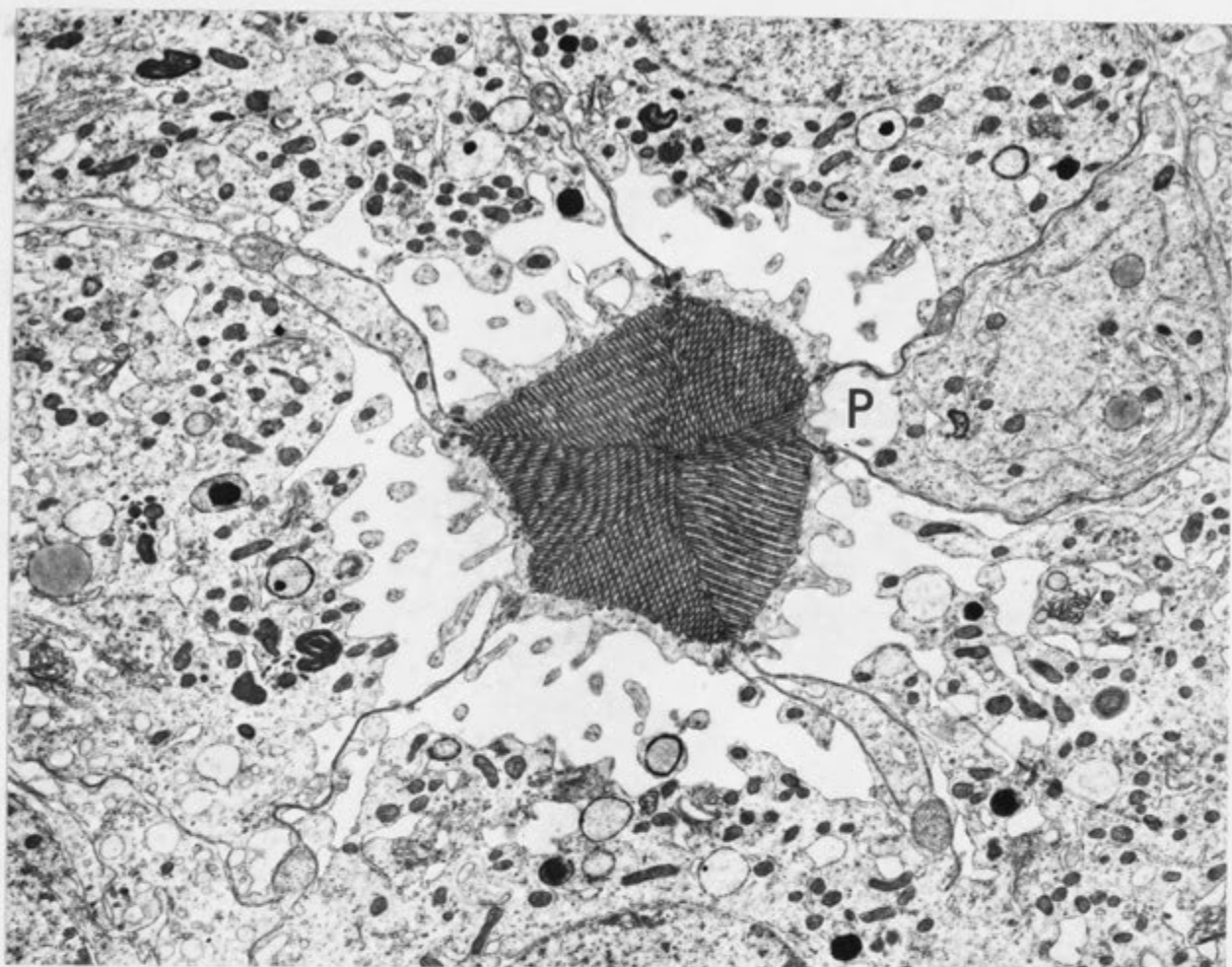


Fig. 4. Transverse sections through the distal part of an ommatidium in a (top) normal night-state, b (bottom) normal day-state. P, peri-rhabdomal palisade. a and b: X 6500.

Table 1. Rhabdom structure during night and day

	Rhabdom Cross-sectional Area ¹ (μm^2)	Microvillar Diameter ² (nm)	Intramembrane Particle Density ² (μm^{-2})
Day	3.6 ± 0.58	68.0 ± 1.55	6401 ± 373
Night	17.0 ± 1.37	66.8 ± 1.19	6089 ± 424
Probability of no difference ³	< 0.001	0.5 (n.s.d)	0.3 (n.s.d)

¹ from Fig. 8a

² means of 30 measurements of different rhabdoms sampled from 4 animals

³ t-test (two tail); n.s.d., no significant difference

Error is ± 2 standard errors of the mean

Cycle a in Fig. 8 shows measurements of rhabdom cross-sectional area in Locusta at various stages throughout a normal daily cycle. During a period of 1 h after the onset of light at "dawn", the cross-sectional area is reduced by 79% from its "night-state" size to its "day-state" size (Table 1; cf. Fig. 2a with 2b, and 4a with 4b), after which there is no significant change until the following dusk. The post-"dawn" reduction is achieved by massive pinocytosis of membrane from the bases of the microvilli (Fig. 5). The basal parts of the microvilli initially become disorganised. Coated pits, which pinch off as coated vesicles, form in the disordered photoreceptor membrane and some quite large segments of membrane are sloughed from the rhabdom. After about 30 min, the rhabdom has resumed an ordered state and membrane continues to be shed by the pinocytosis of coated pits, as in most other arthropods (e.g., Eguchi and Waterman 1967; White 1968; Blest 1978; Martin and Hafner 1981). If exposed to a natural sunrise of gradually increasing light intensity, the microvilli remain strictly aligned throughout the entire dawn period and pinocytotic diminution of the rhabdom proceeds more slowly. At sunrise, about 1 h after the first noticeable light of dawn in the east (mid-summer, 35° latitude), the cross-sectional area of the rhabdoms is midway between night-state and day-state size (20 rhabdoms from each of two animals averaged 9.6 ± 0.49 (2 S.E.) μm^2). By 2 h after sunrise, rhabdoms have reached their normal day-state (Figs. 2b, 4b).

Immediately after the onset of darkness, the night rhabdom is constructed by the assembly of new photoreceptor membrane. During construction the rhabdomeres are characteristically disarrayed (Fig. 6).

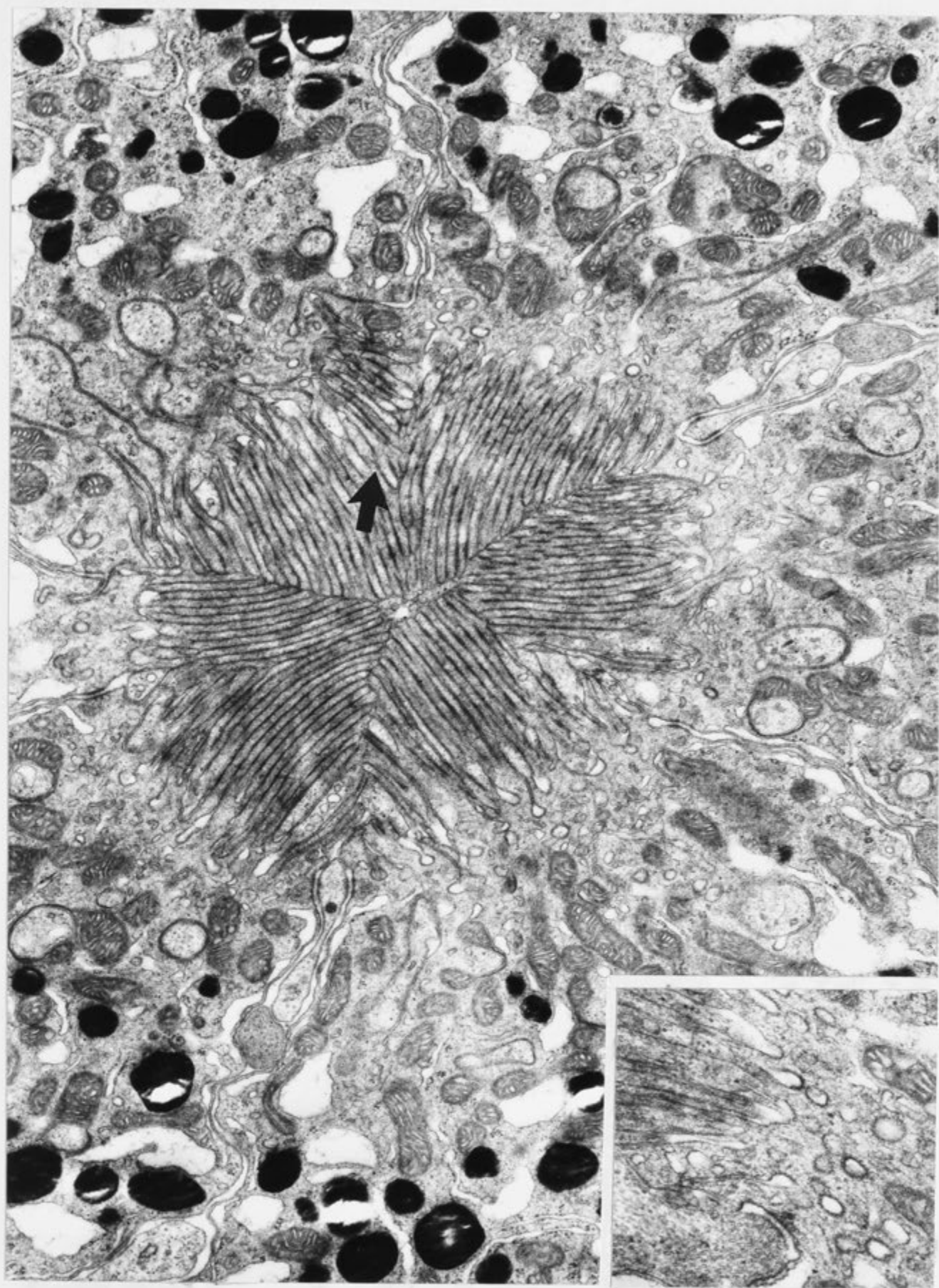


Fig. 5. Rhabdom on normal cycle fixed 15 min after "lights-on". At their bases the microvilli are disordered and vesiculating. The shorter microvilli at one margin of each rhabdomere (e.g., arrow) are shed in toto as rhabdomeral orientation changes. X 17,800. Inset. Higher magnification of vesiculation at the bases of microvilli about 30 min after lights-on. X 32,400.

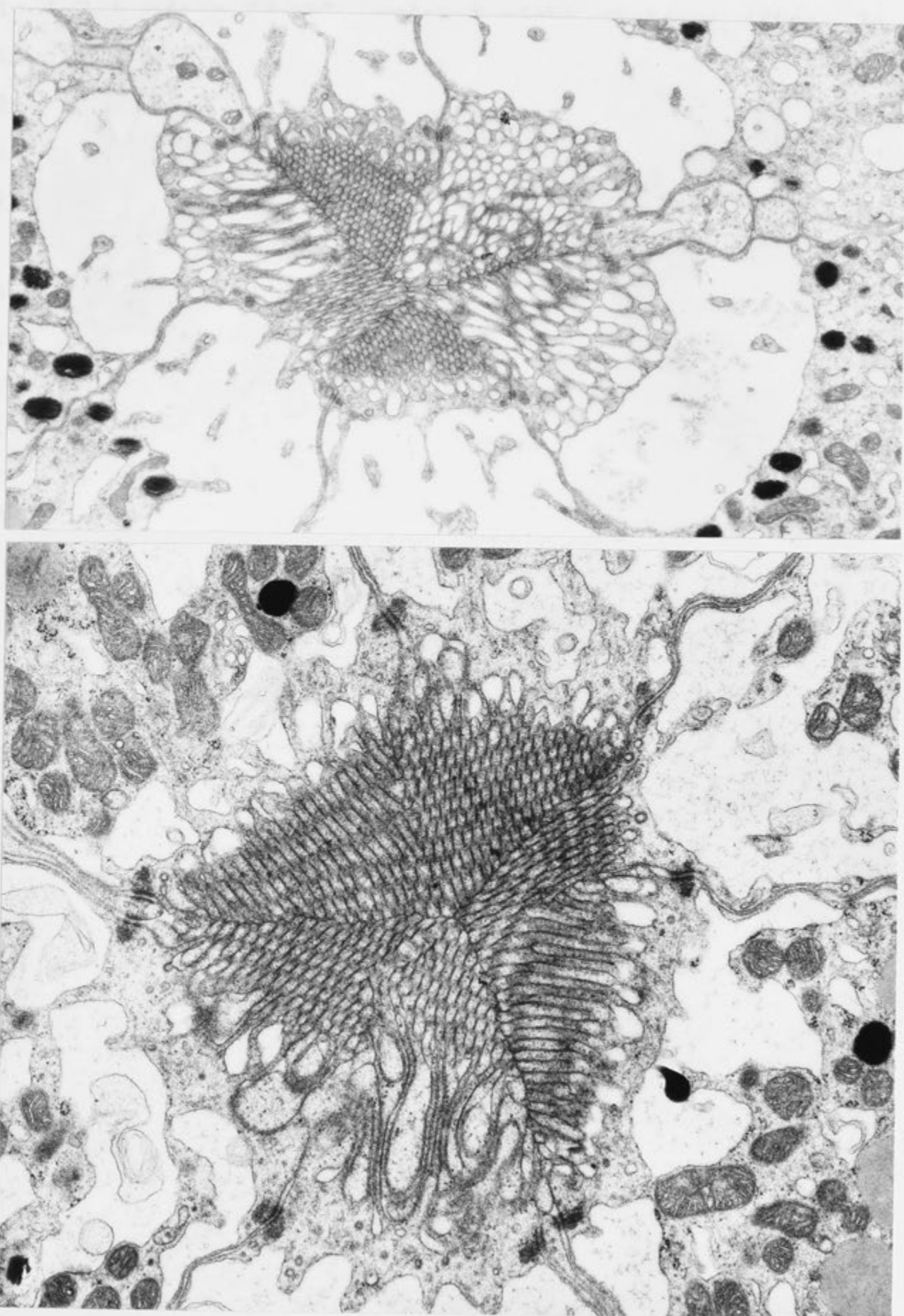


Fig. 6. Disarrayed rhabdomeres during the assembly of new microvillar membrane to form the night rhabdom immediately after "dusk". a (top) Three rhabdomeres are disordered during an early stage of reassembly. X 11,000. b (bottom) Except for two rhabdomeres (lower, left), assembly is practically completed. X 17,000.

By 1-3 h after "dusk", ordered night rhabdoms (Figs. 2a, 4a) are evident, and they remain in this state until dawn. In cycle a (Fig. 8), measurements of rhabdom size after dusk are shown only for ordered rhabdoms in the night-state.

Between night and day, a change in orientation of microvilli in the distal rhabdom is associated with the change in rhabdom size. In the day-state, the distal rhabdom typically comprises six rhabdomeres with pairs of adjacent rhabdomeres having nearly the same microvillar orientation. The result is a triradiate pattern (Fig. 4b) (Wilson et al. 1978). Proximally, this pattern is lost as rhabdomeres twist (Williams 1981 for discussion). In the night rhabdom, however, the triradiate pattern is not present, for rhabdomeres have independent orientations even in the distal rhabdom (Fig. 4a). At dawn, the shorter microvilli at one lateral margin of each night rhabdomere are shed entirely to give rise to the triradiate configuration (Fig. 5).

Microvillar Structure

Only the length and number of microvilli have been found to vary between day and night in Locusta. Firstly, unlike in Limulus (Behrens and Krebs 1976), the mosquito (White 1967), and a crab (Nassel and Waterman 1979), the diameter of the microvilli was constant (Table 1). Secondly, the intramembrane particles found on the P-face of the microvilli did not change in density (Table 1; Fig. 7), and their size appeared to remain at about 8 nm in diameter.

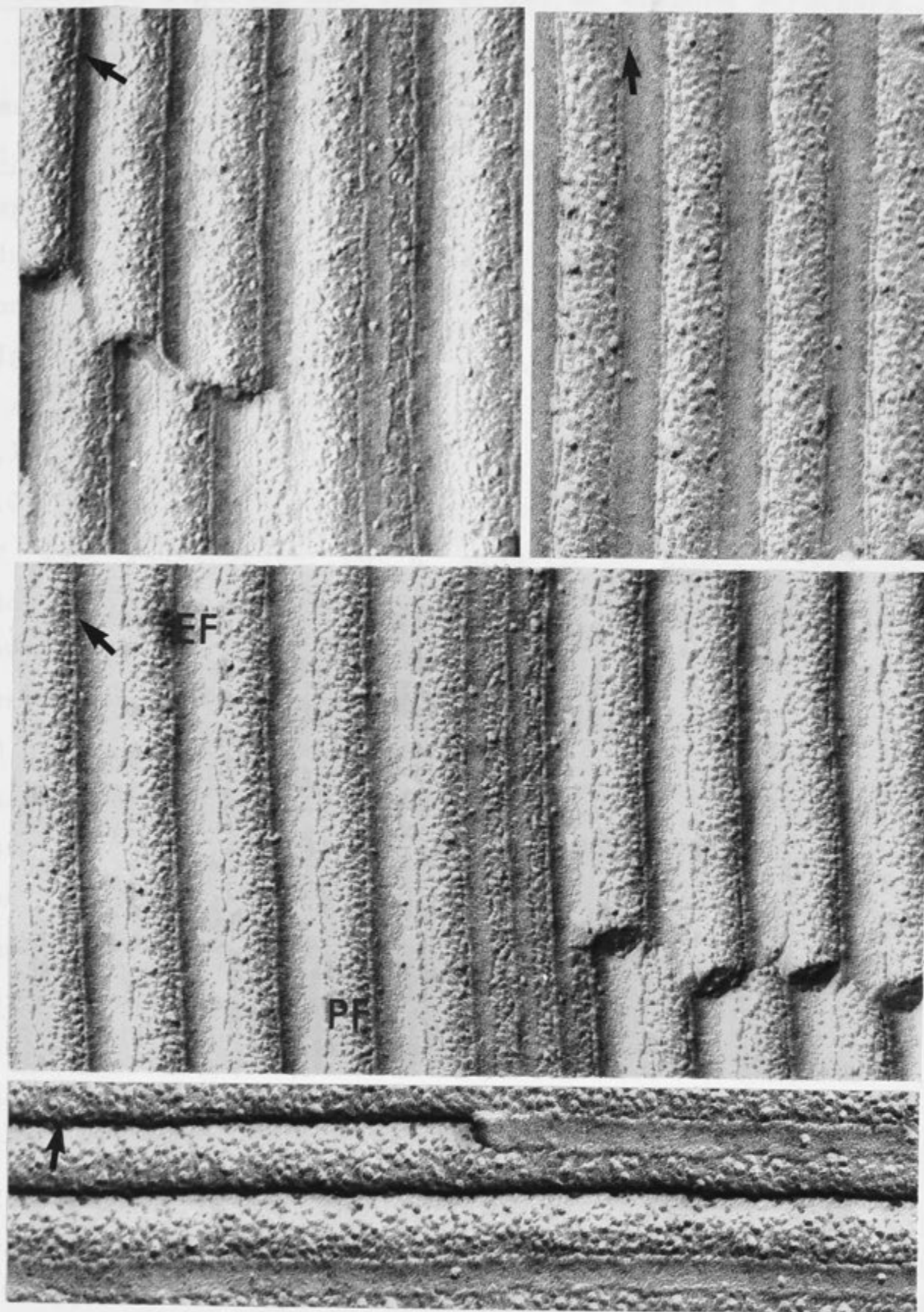


Fig. 7. Electron micrographs of freeze-fracture replicas of photoreceptor microvilli from Valanga. The intramembrane particles on the cytoplasmic face (PF) probably represent mostly rhodopsin molecules, or aggregates thereof. EF, face of outer membrane layer. Arrows indicate direction of platinum coating. a, b (top) fixed at midday on normal light cycle, c (middle), d (bottom) at midnight. a, b, c X 100,000; d X 130,000.

Effects of Lighting and Time of Day on Rhabdom Structure

Fig. 8b-f illustrates the changes in rhabdom size in response to abnormal lighting regimes.

Light Continued After Day (Fig. 8b). Rhabdoms of animals kept in the light and fixed 1, 2 and 4 h after the normal time of "dusk", possessed the distal triradiate configuration, and were significantly smaller than normal night-state rhabdoms ($p < 0.001$), but those fixed 1 and 2 h after 1800 h were significantly larger (mean size: 6.0 ± 0.79 (2 S.E.) μm^2) than the normal day-state ($p < 0.002$). Most rhabdoms in two eyes fixed at 2000 h had one or two rhabdomeres in a disarrayed state (Fig. 9), indicative of assembly of new microvillar membrane. However, large numbers of coated pinocytotic pits and vesicles were present at the bases of the microvilli, and in and around the assembling receptor membrane, implying that much of the newly synthesised microvillar membrane was rapidly being shed, thus maintaining a small rhabdom. A similar process has been described in a crab under this light regime (Stowe 1981).

Darkness Continued After Night (Fig. 8c). The sudden burst of photoreceptor membrane shedding at the time of "dawn" does not occur in the absence of light (Fig. 10), but eventually, in the dark, rhabdom size does decrease. By 1200 h, rhabdoms (mean size: 9.8 ± 1.67 (2 S.E.) μm^2) were found to be significantly smaller than at night ($p < 0.001$), but the distal triradiate configuration was not attained until 2000 h. Occasional coated vesicles and secondary lysosomes, similar to those found in photoreceptor cells fixed after exposure to light at dawn, were observed in photoreceptors fixed on this regime. Hence, the rhabdom diminution that occurs during the day while locusts are held in darkness appears to be achieved

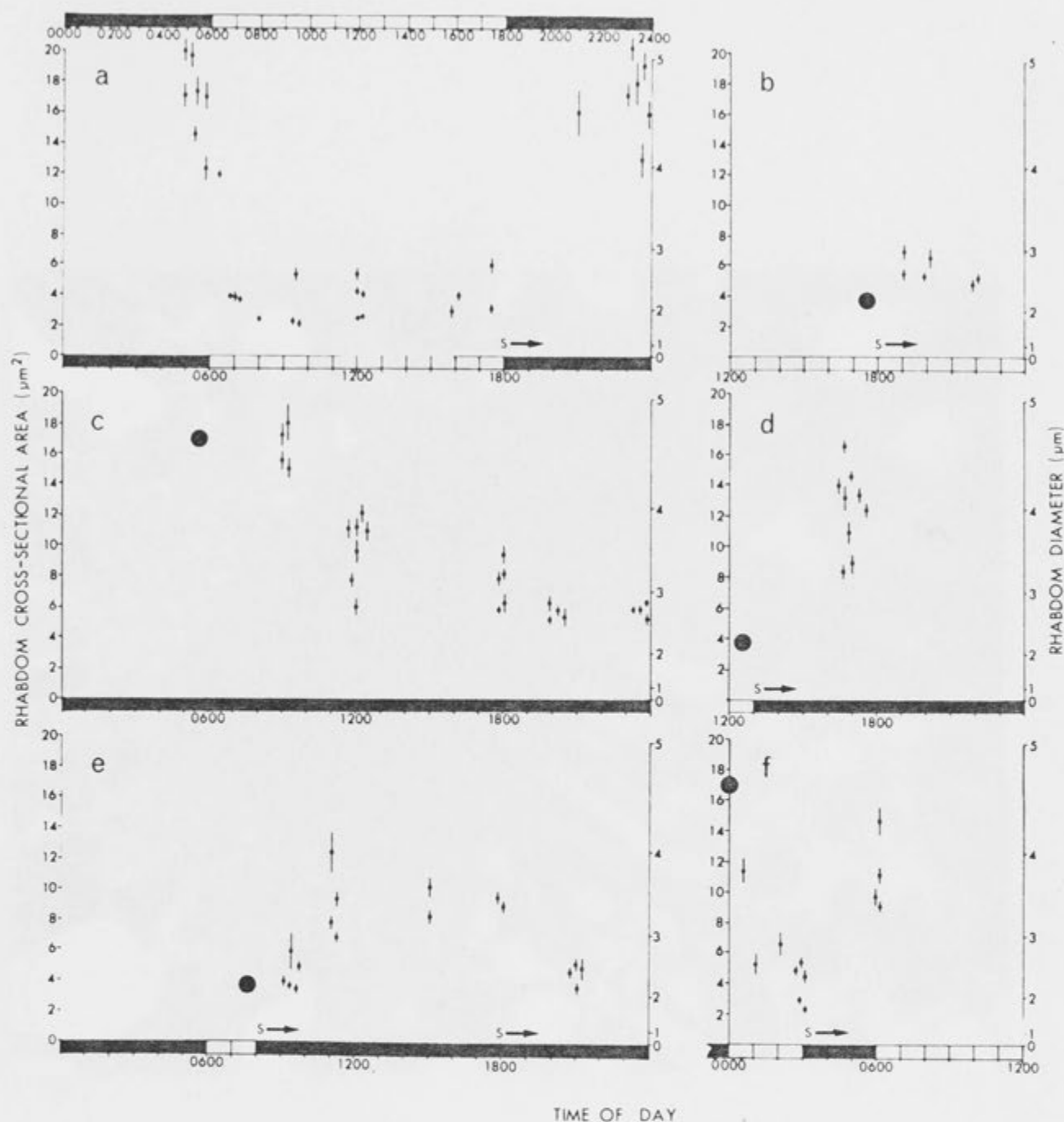


Fig. 8. Cross-sectional area (and diameter for circular shape) of rhabdoms in the central region of a compound eye in *Locusta*, in relation to time of day and lighting conditions. Each point represents the mean size of 15-30 rhabdoms from one animal. Vertical bars extend 2 standard errors of the mean. Large dots represent mean size on the normal cycle (a) before the start of the abnormal lighting. Microvillar assembly (Fig. 6) was observed at times indicated by "S +". For at least 2 weeks prior to the day of fixation, animals were exposed to a 12 h light: 12 h dark cycle (uppermost). Lighting conditions on the experimental day (beginning midnight) were as follows: a normal cycle continued; b light continued into night-time; c darkness during the day; d "dusk" 5 h early; e "dusk" 10 h early; f 3 h light after midnight.

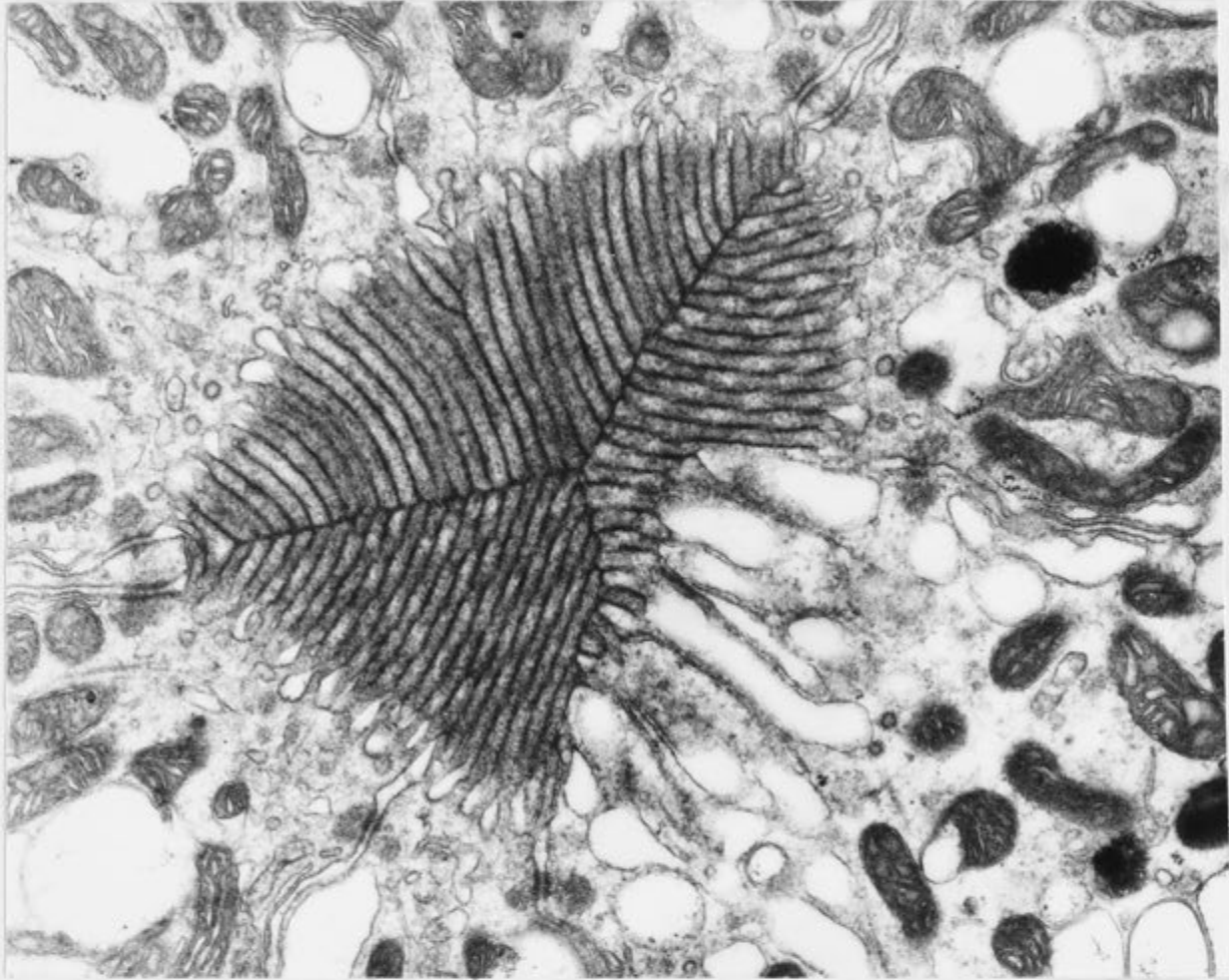


Fig. 9. Rhabdom of Locusta fixed after being kept in light 2 h after normal time of dusk. One rhabdomere is undergoing reassembly. Pinocytotic vesicles are budding off from much of the rhabdom. X 25,000.

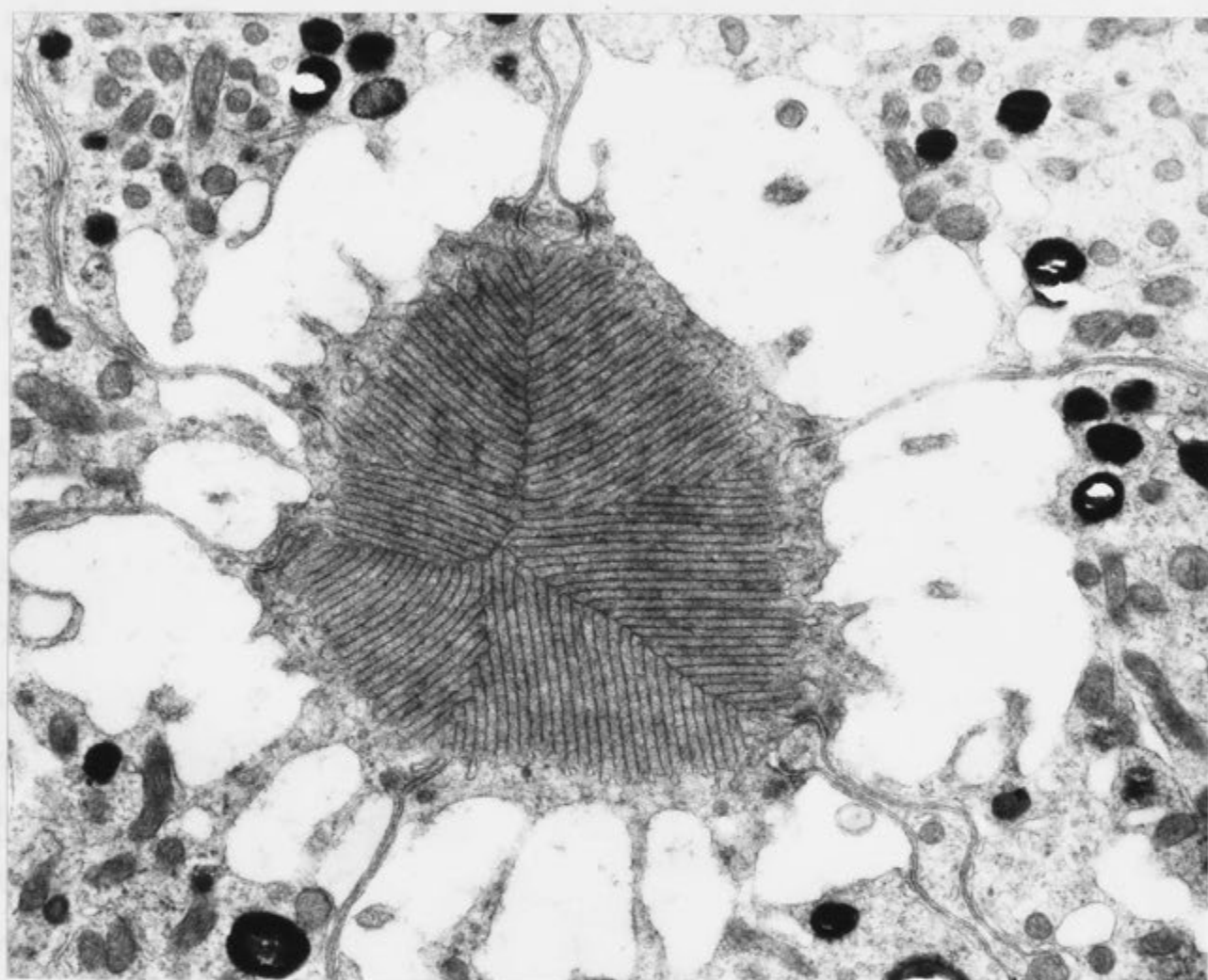


Fig. 10. Rhabdom of Locusta fixed after being held in darkness 6 h after normal time of dawn. X 15,600.

by the same mechanism as normal dawn shedding, albeit on a very much smaller scale.

Darkness Before Dusk (Fig. 8d, e). Assembly of new photoreceptor microvilli occurs whenever a locust is placed in darkness during daytime for a few hours. The ultrastructure of rhabdoms 1-2 h after the onset of darkness on regime d or e was similar to that in normal cycle retinae immediately after "dusk", with many rhabdomeres in the transitional disarrayed state. Nevertheless, a full night-state rhabdom did not result from microvillar assembly at these times. Rhabdoms fixed between 1200h and 1800h on regime e (put in darkness at 0800 h) had lost their distal triradiate configuration but were significantly smaller (mean size: 9.25 ± 1.18 (2 S.E.) μm^2) than in the normal night-state ($p < 0.001$). The size of rhabdoms 3.5-4.5 h after being put in darkness at 1300 h (mean 12.6 ± 1.78 (2 S.E.) μm^2) was also significantly smaller ($p < 0.002$).

Darkness Before and Beyond Time of Dusk (Fig. 8c, e). In two eyes of the four sampled at the normal time of dusk on regime e, rhabdoms (not measured) were found to be reassembling their microvilli. Yet rhabdoms measured after 1800 h on this regime were smaller (mean size: 4.7 ± 0.74 (2 S.E.) μm^2) than those measured before 1800 h (mean size: 9.25 ± 1.18 (2 S.E.) μm^2) ($p < 0.002$). No rhabdoms from the four eyes fixed on regime c at the normal time of dusk were found to be undergoing microvillar assembly, and rhabdoms fixed later still were of a size that could be expected from a continuation of the gradual diminution that had begun almost 12 h earlier (Fig. 8c). Assembly of new microvilli therefore appears to occur at the usual time of dusk on regime e but not c, and on regime e it results in rhabdoms that are reduced in size.

Light At Night (Fig. 8f). When the same daylight-fluorescent lighting used for normal daytime (see Materials and Methods) was switched on at midnight, eyes fixed between 0030 h and 0200 h had many rhabdoms with microvilli that had lost their normal orderly configuration (cf. Horridge et al. 1981) (Fig. 11). Particularly at their bases, membranes of adjacent microvilli collapsed together to form dilated "loops" of membrane. The presence of coated pits in these membranous loops (Fig. 11) suggest that the membrane is being internalised by pinocytosis. About 3 h after the onset of light, the rhabdoms had assumed a configuration and size (mean: 3.9 ± 1.24 (2 S.E.) μm^2) that was indistinguishable from the normal day-state (size: $p = 0.7$).

After being returned to the dark for 3 h after 3 h of light, rhabdom size (mean: 11.3 ± 2.57 (2 S.E.) μm^2) had increased over that immediately prior to darkness, and the distal triradiate configuration was lost, showing that new membrane had been assembled. Rhabdom size was, however, significantly smaller than that of normal night-state ($p < 0.002$). Some rhabdoms had disarrayed rhabdomeres, indicating that they were still in the process of microvillar assembly.

Comparisons with Valanga

Rhabdoms from the central region of the compound eyes of 12 adults of another locust, Valanga irregularis, were examined according to regimes (as in Fig. 8): a (2 day-state, 2 night-state), c (2 at 0900 h, 2 at 1800 h), d (2 at 1730 h), and f (2 at 0300 h). In all cases rhabdom structure was similar to the corresponding Locusta rhabdoms, even approximately to the extent of absolute size of the rhabdom.

Changes in Visual Structure

Rhabdom Structure. Even after 24 h of dark adaptation, rhabdoms and rhabdomeres

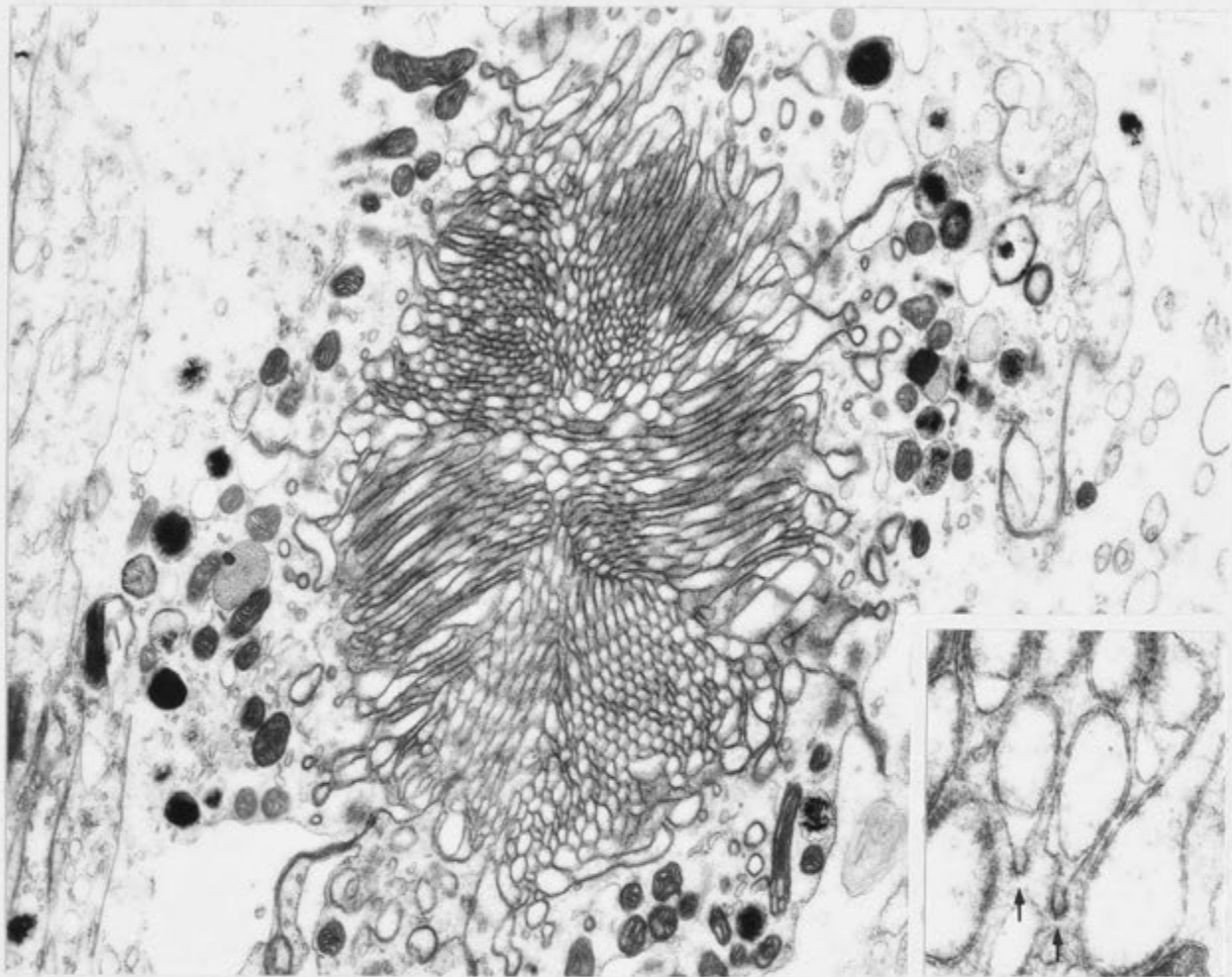


Fig. 11. Rhabdom of Locusta fixed after exposure to 0.5 h light at midnight. X 12,600. Inset. Higher magnification of coated pits (arrows) forming in loops of disordered membrane in a rhabdom after exposure to 2 h light at night. X 37,000.

Discussion

Changes in Ommatidial Structure

Rhabdom Structure. Even after 24 h of dark adaptation, Horridge and Barnard (1965) found the rhabdoms of Locusta migratoria that were reared in constant light to be the same size as those of light-adapted locusts, although they stated that there was a greater "variability of cross-sectional area at the 0.5 per cent level of significance". Horridge et al. (1981) found, however, that the rhabdoms of the locust, Valanga, are larger at night than during the day. The present results show that rhabdom size changes on a daily cycle in Locusta also (Fig. 8a). In addition, it has been shown that (1) rhabdomeral orientation changes; (2) the size of the ommatidial field stop changes in co-ordination with rhabdom size; but (3) the diameter and intramembrane particle density of the microvilli are constant.

Effects on Angular Sensitivity. Unlike in the compound eyes of some other arthropods (e.g., the bug, Lethocerus, Walcott 1971; tipulid fly, Ptilogyna, Williams 1980a; Limulus ventral eye, Behrens 1974 and Miller and Cawthon 1974), the distal end of the rhabdom in Locusta was not found to move a significant distance distally into an out-of-focus position at night. Therefore, given that the function of the dioptric system can be reasonably explained in terms of geometrical optics (McIntyre 1982), the number of photons impinging on the rhabdom from an extended field at a given time is directly dependent on the area of the field-stop. Since the areas of the pigment field-stop and the rhabdom appear to change in tandem, changes in the area of the field-stop are indicated by the more accurately determined changes in

cross-sectional area of the rhabdom. The 4.7-fold increase in cross-sectional area of the rhabdoms at night therefore indicates a 4.7-fold increase in the number of photons entering the rhabdom from an extended source. Because rhodopsin concentration is inferred to remain constant between day and night (see below), the proportion of captured photons absorbed is unchanged for a given rhodopsin/metarhodopsin equilibrium. Therefore, the photon absorption by a rhabdom from an extended source should be 4.7 times greater in a dark-adapted eye at night than in an eye dark-adapted during the day for 15-20 min: i.e., before the assembly of new membrane, but after the formation of the palisade (Horridge and Barnard 1965).

Because this increase in photon absorption is achieved by increasing the angular acceptance of the rhabdoms, it is at the expense of the spatial acuity of the eye. "Trading off" a fine visual angle to increase sensitivity, whether at the photoreceptor level or at a higher level (e.g., Barlow et al. 1957), is a common strategy of dark adaptation. Its advantage has been discussed by many authors (see Snyder 1979 for review).

Implications for Photoreceptor Membrane Turnover. That some arthropods with constant-size rhabdoms appear to turn over photoreceptor membrane (PRM) (Blest and Maples 1979; Williams 1982), and indeed that most of all surface membranes turn over (Poste and Nicholson 1976), implies that the variable-size rhabdom has evolved from a basic cellular mechanism. To achieve a significant variation in size, however, a rhabdom must daily turn over a large proportion of its membrane, in addition to having the shedding and assembly phases of its turnover at least partially separated. Turnover of so much PRM would seem to be costly in terms of energy requirements for PRM

synthesis and degradation. Blest (1978) considered that although Dinopis should benefit from an increase in visual sensitivity due to more PRM at night than during the day, this increase is small and the primary reason for turning over PRM so quickly was probably not in order to effect changes in sensitivity. The present results from Locusta support this viewpoint.

In Locusta, sensitivity is controlled by the field-stop of pigment and not rhabdom size. Consequently, it should make little optical difference if the larger night rhabdom was kept during the day: the smaller angular acceptance of the rhabdom would still be obtained by the narrowing of the field-stop. This argument also holds for many other insects (e.g., Lethocerus, Walcott 1971; Ptilogyna, Williams 1980a), many crustaceans (e.g., Stowe 1980a) and Limulus (Behrens 1974; Miller and Cawthon 1974), all of which are capable of stopping down light entering their rhabdoms from an extended field by several log units with screening pigment, and yet still have variable-size rhabdoms. The variable-size rhabdom, with its large daily amount of turnover, therefore, must have resulted from some demand other than that of light/dark adaptation. Perhaps, it is more economical for variable-size rhabdoms to turn over large amounts of PRM each day than to maintain PRM that is not essential to the performing eye. The rapid turnover of variable-size rhabdoms contrasts with the comparatively slow turnover and constant size (cf. Basinger et al. 1976) of the rod outer segments of vertebrates: the life-span of rod outer segment discs is 10 days in rats and mice and considerably longer in amphibians and goldfish (Young 1967; for review see Kinney and Fisher 1978). The daily rate of bulk turnover of PRM in constant-size rhabdoms is not known

(cf. Williams 1982).

Effects on Polarisation Sensitivity. The change in orientation of the dichroic microvilli in the distal rhabdom between day and night states should change the vector of polarization sensitivity of a photoreceptor cell by up to 15° . This change has particular relevance to a current theory on electrical coupling between locust photoreceptors. When recording intracellularly from a receptor, Lillywhite (1978) found very small discrete depolarisations in membrane potential, which he called "small bumps" and attributed to single photon absorptions by a receptor neighbouring the impaled cell. Lillywhite hypothesized that these photon absorptions were recorded in the impaled cell because neighbouring photoreceptors are naturally electrically coupled. In support he reported that polarisation sensitivity curves for small bumps matched what would be expected for responses from neighbouring cells, based on the triradiate rhabdom configuration. However, his locusts were dark-adapted for several hours in the afternoon, and therefore probably had night-state rhabdoms, lacking the distal triradiate configuration.

In view of the present findings, the question of interreceptor coupling in the locust retina should be re-examined.

Constancy of Rhodopsin Concentration

It is now considered that the intramembrane particles on the P-face of photoreceptor microvilli probably represent mostly rhodopsin molecules, or at least aggregates of the photopigment molecule (see discussions by Fernandez and Nickel 1976; Nickel and Menzel 1976). Accordingly, because the intramembrane particle size and

density in the freeze-fracture replicas of day- and night-state locust rhabdoms are the same, it can be concluded that the rhodopsin concentration is probably unchanged between these two states. In the bull ant, Nickel and Menzel (1976) also found no change in intramembrane particle density between dark- and light-adapted rhabdoms.

Horridge and Blest (1980) noted that if rhodopsin was inserted into preformed membrane during synthesis of PRM, rhodopsin concentration could be manipulated according to day or night, and PRM would thus be enriched with rhodopsin during dark adaptation in order to increase photon absorption efficiency. Their prediction is not supported by the present results, or those of Nickel and Menzel (1976). The possibility of this mechanism of PRM synthesis should not be ruled out, however. The rhabdoms of many arthropods, such as Locusta, are long (300 μm in adult Locusta), so that their absorption efficiency of light during the day is already extremely high at the maximally absorbed wavelength (cf. Kirschfeld 1969), and any increase in rhodopsin density could only significantly increase the absorption of photons whose wavelength is distant from peak wavelength (450-500nm in Locusta: Lillywhite 1978). Unless this effect is important, it would be unlikely that, in arthropods with long rhabdoms, rhodopsin would be increased at night over that during the day, even if the means to do so were available.

Photoreceptor Membrane Turnover and Its Control

On a normal light cycle, microvillar formation in Locusta is discontinuous, occurring over only a few hours after dusk. Since no reduction in size was detected in the day- or night-state rhabdoms once they were

established, any basal rate of shedding must be low. Accordingly, microvillar assembly and shedding are practically completely separated, and a change in rhabdom size usually indicates the occurrence of one of these two phases of PRM turnover. This situation differs from that in the larval mosquito eye, where rhabdom size is the result of a certain balance between shedding, which ceases only in darkness, and continual assembly (White and Lord 1975; White et al. 1980).

Accompanied by observations on cytological changes to indicate when microvillar assembly was in process (Fig. 6), changes in the size of the locust rhabdom (Fig. 8) indicate the following: (1) Light induces shedding, and can elicit complete shedding even at night. Shedding also occurs slowly in darkness after the time of dawn. The rapid shedding that Horridge et al. (1981) believed to occur in locusts held in darkness throughout and after dawn has not been confirmed by the present study. (2) Microvillar assembly is triggered by the onset of darkness. It also occurs at the normal time of dusk without a change in ambient lighting, provided there has been some light during the day.

Shedding. On a normal light cycle, shedding of variable-size rhabdoms (Blest 1978) and rod outer segments (LaVail 1976; Basinger et al. 1976) peaks at dawn. Now it is recognized that light is essential for full dawn shedding of rhabdoms in representatives from all major groups of arthropods, and of rod outer segments in amphibians but not rats (Table 2). Endogenous effects on shedding are apparent, however. The shedding that occurs in darkness during daytime (Table 2) probably results from an endogenous stimulus. Even in frogs, where no spontaneous shedding is evident during one day without light, after

Table 2. Morning shedding in darkness.

Animal	Time Sampled after "dawn"	% Normal Shedding ¹	Reference
<u>Dinopis</u> (spider)	2h	"none"	Blest (1978)
<u>Limulus</u>	not stated	"none"	Chamberlain & Barlow (1979)
<u>Grapsus</u> (crab)	4h	47%	Fig. 6 in Nassel & Waterman (1979)
<u>Procambarus</u> (crayfish)	1h	30%	Fig. 8 in Hafner et al. (1980)
<u>Locusta</u> (insect)	3h 6h 12h	0% 44% 61%	Fig. 8c in present report
<u>Rana</u> (frog)	6 & 14h	"none"	Basinger et al. (1976)
<u>Xenopus</u> (tadpole)	4h 8h	20% 42%	Figs. 18 & 22 in Besharse et al. (1977a)
Albino rat	1 - 9h	100%	LaVail (1976)

¹ Shedding determined in invertebrates by decrease in rhabdom diameter, and in vertebrates by increase in number of phagosomes from rod outer segments per unit length of the retinal pigment epithelium.

constant darkness for several days some outer segments eventually shed (Basinger et al. 1976). In Limulus, efferent nervous input of the retina has been identified as a source of endogenous stimulus. Although light is essential for dawn shedding, the efferent input must also be intact (Chamberlain and Barlow 1979). In rats, rod outer segment shedding follows a circadian rhythm (LaVail 1980). Horridge et al. (1981) emphasised the endogenous component of shedding in the locust, Valanga; however, their conclusion seems to be based on a misinterpretation. Their figure (Fig. 1D), which they state shows a rhabdom spontaneously shedding after being kept in darkness for 4 h past the normal time of dawn, depicts entire microvilli that are dilated and somewhat disarrayed, and thus is not typical of a locust rhabdom undergoing normal membrane shedding; in fact, it appears more like a rhabdom assembling new microvilli (cf. Fig. 6a). The four Valanga eyes and the Locusta eyes (Fig. 8c) that were sampled in the present study after they had been held in darkness throughout and after dawn, had their microvilli in the same ordered configuration as in the normal night-state (Figs. 9, 12). In partial agreement with Horridge et al., it was found that some shedding does occur in locusts during the morning in darkness (Fig. 8c; Table 2); but there was no indication that this shedding was achieved by any other method than a smaller and more protracted version of the normal shedding mechanism, involving pinocytosis from the bases of the microvilli. A second observation, reported by Horridge et al. to show endogenous control of shedding, was that rhabdoms exposed to light at night remained larger than normal day-state rhabdoms. In the present study, no significant difference was found between the size of rhabdoms after 3 h under the

same light intensity during the day or night in Locusta (Fig. 8f) ($p = 0.7$) or Valanga. Nevertheless, changes in rhabdom structure induced by light at night are quite distinct from those at "dawn" (cf. Figs. 5, 11). Similarly, in Dinopis, during the breakdown of PRM after exposure to light at night normal internalisation by pinocytosis does not take place (Blest 1980).

Therefore, shedding of PRM in locusts, as in many animals, is induced by light, but there is also clearly some endogenous control.

Microvillar Assembly. Assembly of new PRM in mosquitoes (White and Lord 1975; Brammer et al. 1978) and amphibians (Besharse et al. 1977b) proceeds more gradually than in a locust. In locusts this process is similar to that in the crab, Leptograpsus, where it is obviously discontinuous, and involves the dissolution of the existing rhabdom, followed by assembly of entirely new microvilli (Stowe 1980b). A major distinction between the crab and locust appears to be that new membrane forms directly from the endoplasmic reticulum in crab (Stowe 1980b), whereas in the locust, the Golgi apparatus is implicated as the immediate precursor of microvillar membrane (own unpublished observations).

Microvillar assembly occurred at 1800 h without any change in lighting and in near constant temperature and humidity, when there had been some light during the day: it occurred on regime b (constant light throughout dusk) and e (2 h light from dawn followed by darkness), but not on c (darkness all day). That assembly is initiated at the normal time of dusk under these conditions indicates that it is under some endogenous control, although the strongest evidence for this view would involve showing a circadian nature (cf. Brady 1974).

As in crabs (Nassel and Waterman 1979; Stowe 1981) and Dinopis (Blest 1978), the assembly of new microvilli was found to be triggered by the premature onset of darkness, but a full-sized night rhabdom was not achieved if the locust was exposed to darkness much earlier than the normal time of dusk. Stowe (1981) noted in Leptograpsus that the smaller rhabdom resulting from an "early dusk" did not seem to be due to an inability to carry out any particular synthetic step, and was likely to be the result of a shortage of material(s). At 2100 h on regime e (when the eye had been in darkness since morning), rhabdom size (from a preliminary sample) reduced from that before 1800 h ($p < 0.002$) apparently by the assembly of new microvilli. This reduction could result from a reassembly program involving the disposal of the existing rhabdom and assembly of a new one (cf. Stowe 1980b), combined with a low supply of materials for the assembly of new microvilli at the time of dusk: microvillar assembly had already occurred once since the previous dusk.

In conclusion, initiation of microvillar assembly, like that of shedding, is influenced by ambient lighting and an element of endogenous control. In addition, the extent of assembly appears to be dependent on a prior build-up of materials.

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CHAPTER VIII

PHOTORECEPTOR MEMBRANE SHEDDING AND ASSEMBLY
CAN BE INITIATED LOCALLY WITHIN AN INSECT EYE

David S. Williams

Abstract. Photoreceptors of locust compound eyes add new receptor membrane at dusk, and shed membrane at dawn. When part of an eye is masked before dusk, premature assembly of new membrane is initiated in the masked ommatidia but not adjacent unmasked ommatidia. Similarly, masking some ommatidia just prior to dawn prevents normal shedding only in the masked ommatidia. Therefore the shedding and assembly phases of photoreceptor membrane turnover can be initiated by a change in the state of illumination of individual ommatidia.

Photoreceptor membrane (PRM) turns over according to a daily cycle in vertebrates (1) and arthropods (2). During turnover, the shedding of the rod outer segments of vertebrates and the rhabdomeres of arthropods normally peaks at dawn, under the influence of both the onset of light and endogenous factors (3-5). Assembly of new rod outer segment discs is also greatest during the morning (6). However, in some arthropods, assembly of new microvillar PRM is restricted to a few hours just after dusk, and, like shedding, appears to be controlled both endogenously and by a change in ambient lighting (2,5,7). Attempts to localise the control mechanisms of PRM turnover have shown that in a variety of animals both shedding and assembly are controlled in each eye independent of the other. Shedding in the frog, Rana, is prevented in one eye if it is masked at dawn without affecting shedding in the unmasked eye (8). When one eye

of a crab is masked in the afternoon, new PRM is soon assembled in that eye but not in the other (7). Initiation of shedding, as well as synchronisation of its circadian timing by the central nervous system, occurs unilaterally in rat eyes (9). Similarly Limulus lateral eyes are affected independently by efferent input that is necessary for normal turnover (4). Lastly, in vitro isolation of single eyes does not prevent light-stimulated shedding and assembly of PRM in Xenopus (10), or assembly of PRM, when initiated by premature darkness in a locust or at the time of dusk without darkness in a crab (11). Nevertheless, the extent of autonomy within a single retina is not known.

In the present experiments, I have attempted to localise the control of PRM shedding and assembly to within discrete regions of single locust compound eyes. Whereas all the photoreceptors of a vertebrate eye share one lens system, the compound eyes of most diurnal insects are composed of many optically isolated units, known as ommatidia. I have exploited this arrangement by using simple masking experiments to see if shedding or assembly can be initiated locally in the retina, without affecting adjacent regions.

Each locust ommatidium has 8 receptor cells, which contribute to a rhabdom of photoreceptive microvilli and share the same visual field (12). As in many arthropods, locust rhabdoms vary in size as a result of daily turnover; shedding reduces their cross-sectional area by 4 to 5-fold at dawn, while assembly increases it by a corresponding amount at dusk (5). A change in rhabdom cross-sectional area is therefore a convenient and clear indication of the occurrence of PRM shedding or assembly.

When locusts are placed in darkness 5 hours prior to

the normal time of dusk, premature initiation of the assembly of new PRM increases rhabdom cross-sectional area to about $3/4$ that of the normal night-state (5). In the first experiment, young adult Valanga irregularis, which had been reared under cyclic lighting (13), had part of one of their large, lightly-coloured eyes covered with black tape 5 hours before "dusk". Half the eye (3 animals) or a 0.3-mm horizontal strip across the middle of the eye (3 animals) was masked. The other eye was left unmasked and served as control. For additional control, 3 animals were placed entirely in the dark. Eyes were fixed for electron microscopy 4 hours after masking (14). Examination of sections of the eyes showed that the rhabdoms of masked ommatidia, and only those of the masked ommatidia, had enlarged, and therefore undergone assembly of new PRM (Figs. 1 and 2a). Another indicator of the occurrence of PRM assembly is a characteristic disarray of the rhabdoms during the process of assembly (5). In additional eyes, fixed 0.5-1 hour after masking, the rhabdoms of masked ommatidia were found in this state.

When locusts are maintained in darkness past dawn, rhabdom size diminishes gradually, but there is no significant change in size for the first few hours (5). A second experiment used this fact to localise initiation of normal shedding. Seven animals were kept overnight under a dim red safelight (Kodak 1A, 15 W bulb). Just prior to exposure to light at "dawn" 4 animals had half of one eye masked. Again the unmasked eye served as a control, as did one eye from each of 3 animals that were not exposed to "dawn". Eyes were fixed 1 hour after "dawn". Invariably, the rhabdoms of ommatidia that were not masked had shed their membrane and were smaller than those of masked ommatidia, which were still in a night-state (Fig.

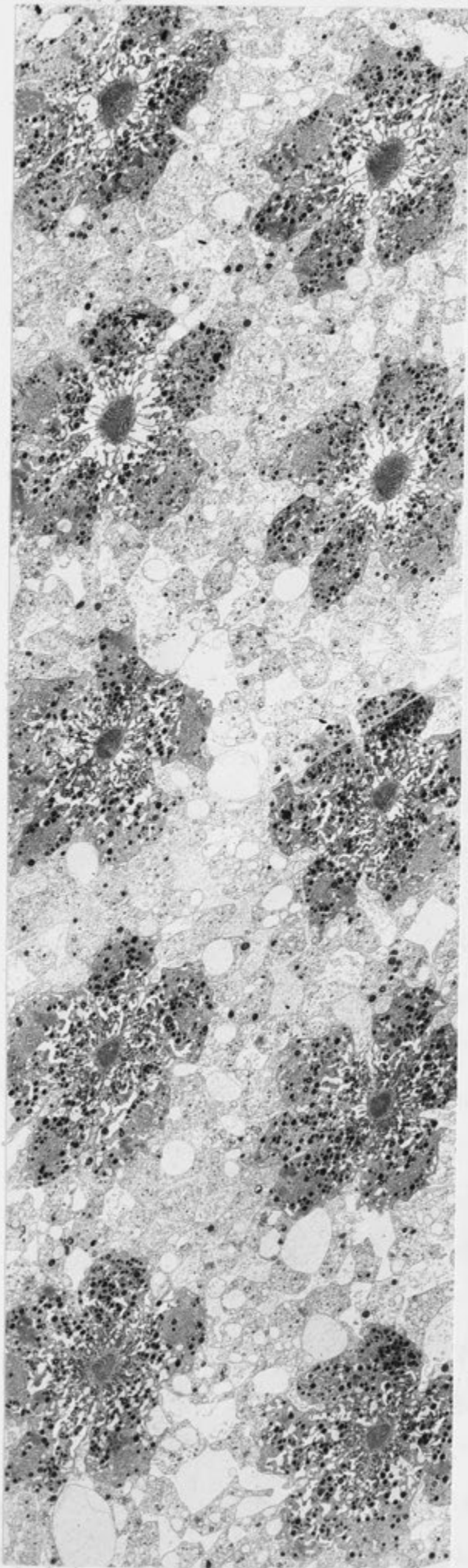
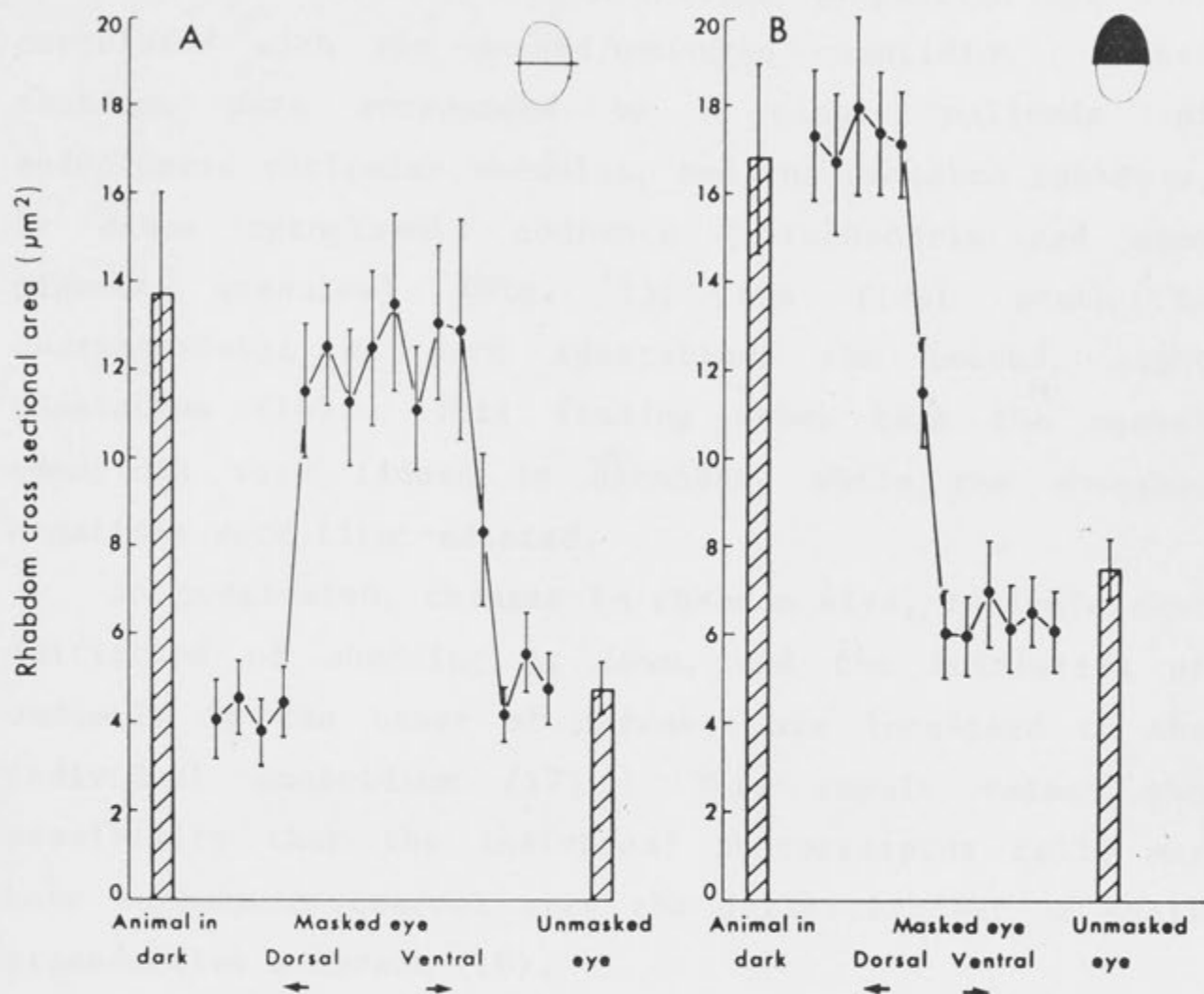


Fig. 1. Electron micrograph of a transverse section of ommatidia across the boundary between masked (bottom) and unmasked (top) parts of an eye. The ventral half of the eye was masked with tape 5 hours before dusk, and the eye was fixed 4 hours later. Over the central region of a locust compound eye, rhabdom size is normally the same among different ommatidia. Masking, however, has induced the 4 rhabdoms on the right to enlarge (they average $13\mu\text{m}^2$ cross-sectional area) by assembling new microvillar membrane. The 4 rhabdoms on the left have remained in the day-state (average cross-sectional area is $5.3\mu\text{m}^2$). The 2 central rhabdoms are possibly in an intermediate state ($6.5\mu\text{m}^2$). The masked rhabdoms are surrounded by a "palisade" of endoplasmic reticular vacuoles. X 1200

Fig. 2. (over page) Graphs of rhabdom cross-sectional area across the boundaries between masked and unmasked regions of an eye. Each point represents the mean of 5 rhabdoms from a horizontal row of ommatidia. The histograms represent measurements of controls. The mean size of 30 rhabdoms sampled from the unmasked eye contralateral to the masked one is on the right. On the left is the mean of 30 rhabdoms measured from 3 animals (10 rhabdoms from 1 eye of each animal) that were placed in darkness (a) or retained in dim red light (b) during the period that the other animals were masked. All bars extend ± 1 standard deviation. Measurements were made from a transverse section taken 20-30 μm from the distal ends of the rhabdoms, where their diameter is nearly uniform (5). The centers of the x-axes of the graphs correspond to the centers of the sections. The interommatidial angle along the vertical axis of the eye is only about 1° , therefore all rhabdoms were considered to be in true transverse section. a The eye was masked with a 0.3-mm horizontal strip of black tape 5 hours before dusk and fixed after 4 hours. A 0.3-mm strip should cover 8-10 horizontal rows of facets. This number corresponds to the number of ommatidia affected by the masking. Masked rhabdoms underwent assembly of new membrane and thus enlarged to the size of rhabdoms in locusts that were placed entirely in darkness (left histogram). In contrast, adjacent unmasked rhabdoms remained at a day-state size, comparable to that of rhabdoms in the other eye, which was left completely unmasked (right histogram). b The dorsal half of the eye was masked just prior to dawn, and the eye fixed 1 hour after "dawn". Unmasked rhabdoms shed their membrane and thus diminished in size, while the masked rhabdoms remained in the night-state, comparable to the



rhabdoms of animals that were not exposed to light at dawn (left histogram). Localisation of the effects of masking, as shown by a and b, were also found with a vertical masked/unmasked boundary, formed by masking the anterior or posterior halves of eyes.

2b). In addition to a smaller rhabdom size, the characteristic secondary lysosomal products of shedding (15) were evident in the photoreceptor cells of the unmasked ommatidia, and absent in the others.

A further effect of dark/light adaptation was also correlated with the masked/unmasked ommatidia. Masked rhabdoms were surrounded by a clear "palisade" of endoplasmic reticular vacuoles, and the unmasked rhabdoms, by dense cytoplasmic contents (mitochondria and some pigment granules) (Fig. 1); the first state is characteristic of dark adaptation, the second, light adaptation (16). This finding shows that the masked ommatidia were indeed in darkness, while the unmasked ommatidia were light-adapted.

In conclusion, changes in rhabdom size, indicate that initiation of shedding at dawn, and the initiation of assembly by the onset of darkness are localised to the individual ommatidium (17). This result raises the possibility that the individual photoreceptor cells may have autonomous control over the daily turnover of their transductive membrane (18).

Changes in the sensitivity of photoreceptors and the position of photoreceptor cell pigment granules during dark-light adaptation in arthropods are localised effects (19). These changes appear to be mediated through changes in intracellular Ca^{++} concentration (20). Since PRM assembly and shedding are likewise local events and associated with dark and light adaptation, perhaps they are controlled by the same signal.

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electrically coupled [P.G. Lillywhite, J. Comp. Physiol. 125, 13 (1978)]. For a description of ommatidial structure see M. Wilson, P. Garrard, S. McGinness, Cell Tissue Res. 195, 205 (1978).

13. Lights-on ("dawn") was at 0600 h, lights-off ("dusk") at 1800 h. Illuminance from daylight-fluorescent plus incandescent lighting was 1000 lux in the centre of each white-sided cage (36 x 36 x 46 cm). Temperature outside the cage was 20-35°C. Animals were fed bran and wheat. At least 24 hours prior to fixation (i.e. including the period when eyes were masked), animals were kept at $25 \pm 2^\circ\text{C}$ and $48 \pm 2\%$ humidity, under the same cyclic lighting as that used during rearing.
14. Prior to primary fixation, dissection was limited to one or two slices through the surface of the eye around the central region, where interommatidial angle is constant. Whole heads were then quickly immersed in primary fixative: 2.5% glutaraldehyde + 2% paraformaldehyde buffered in 0.08 M sodium dihydrogen orthophosphate-NaOH plus 0.06 M D-glucose. After 3-12 hours, the eyes were trimmed, osmicated, dehydrated, and embedded in Araldite. Sections for electron microscopy were stained with uranyl acetate and lead citrate. They were cut so that the ommatidia at the boundary between masked and unmasked areas were in transverse section. Measurements of rhabdom cross-sectional areas were made with a Kontron MOP-AM03 image analyser, from electron micrographs magnified 3300 times.
15. Shed membrane aggregates as vesicles in multivesicular bodies, which degrade through multilamellar bodies to residual bodies (5). This pathway is common to most arthropods: E. Eguchi and T.H. Waterman, Cell Tissue

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 21. Helpful comments on manuscript drafts were provided particularly by Simon Laughlin, Sally Blest and David Blest.

CHAPTER IX

CHANGES OF PHOTORECEPTOR PERFORMANCE ASSOCIATED WITH THE DAILY TURNOVER OF PHOTORECEPTOR MEMBRANE IN LOCUSTS

David S. Williams

Summary. 1. Intracellular recordings were made of receptor responses in the central region of the compound eyes of the locusts, Valanga and Locusta. The animals were maintained on their usual daily light cycle, and recordings were made at times known from previous anatomical studies to coincide with changes in ommatidial structure (Tunstall and Horridge 1967; Horridge et al. 1981; Williams 1982a). Anatomical checks were made of the areas of the retina from which recordings had been made.

2. Angular acceptance at 50% sensitivity in Valanga and Locusta respectively increased from 1.7° and 1.9° when light-adapted during the day, to 2.7° and 2.8° when dark-adapted for 10-15 minutes after "dusk", to 4.7° and 4.9° in a fully night-adapted state. It then decreased to 2.05° and 1.9° when light-adapted for 2 hours after "dawn", and increased to 2.8° and 2.9° after a further 20 minutes dark adaptation (Table 1). Dark-adapted values were measured by exposing the cells to very dim light and counting the quantum bumps (Fig. 1), thus ensuring that there were no light-adaptive effects from the stimulus.

3. Sensitivity to an extended source increased in both species by at least 1 log unit during the first 1-3 hours after "dusk" before reaching its maximum (Fig. 4). The last 0.6 log units of this increment (3.9-fold increase) is attributed to the enlargement of the field stop and rhabdom. After 2 hours light from "dawn", which induces diminution of the rhabdom and field stop to their day sizes, followed by 20 minutes of dark adaptation,

cells were 3.8 times (data from Valanga and Locusta grouped) less sensitive to the extended source than they were at night.

4. No change of spectral sensitivity (Fig. 2), bump latency (Fig. 5), or sensitivity to a point source on-axis (Fig. 3) was detected between the night and day states. The last indicates that the absorption efficiency of the rhabdom is constant, and the Airy disc is smaller than the day-state field stop.

5. The daily changes of sensitivity to an extended source are consonant with the extent of the changes in the field-stop size, together with a constant absorption efficiency of the rhabdom.

6. The increased light capture from the environment, resulting from opening up the rhabdom's acceptance at night, is obtained at the expense of spatial acuity. At night, the angular acceptance at 50% sensitivity becomes more than twice the interommatidial angle ($\Delta\phi$) (Fig. 6). As a result, discrimination of two points spaced at $2\Delta\phi$, which is possible during the day, becomes impossible at night, according to the Rayleigh criterion.

Introduction

Physiological changes between day and night in compound eyes were first recorded by electroretinogram measurements, which emphasised changes of sensitivity according to a circadian rhythm (e.g., beetle, Jahn and Wulff 1943; butterflies, Swihart 1963). Intracellular recordings later showed that sensitivity, in particular the angular sensitivity function of single photoreceptors differed between light and dark adaptation (e.g., Tunstall and Horridge 1967; Walcott 1971). Consideration of an

endogenous rhythm led other workers to measure differences, which were often greater, between day and night (beetle, Meyer-Rochow and Horridge 1975; crabs, Leggett 1978, Stowe 1980a; mantid, Rossel 1979; mantid and locust, Horridge et al. 1981; Limulus, Barlow et al. 1980;)

The changes in sensitivity have usually been explained in terms of movements of screening pigment. Recently, however, changes associated with the turnover of photoreceptor membrane have been held partly responsible for variations of angular sensitivity (crab, Stowe 1980a; locust and mantid, Horridge et al. 1981). In a number of compound eyes, rhabdom size fluctuates according to the daily cycle of photoreceptor membrane turnover. The locust compound eye is one example (Horridge et al. 1981). In locusts, the cross-sectional area of the distal rhabdom decreases at dawn because of the shedding of photoreceptor membrane by pinocytosis. It increases at dusk as new rhabdomeric membrane is assembled en masse. A change in the cross-sectional area of the ommatidial field stop, which is effected by the primary pigment cells situated immediately distal to the rhabdom, occurs in tandem with the change of rhabdom size (Williams 1982a). A previous study measured the angular sensitivity and intensity/response functions of single locust photoreceptors in relation to photoreceptor membrane turnover (Horridge et al. 1981). In this study, however, the daily cycle was not closely followed. Nightfall was given 5 hours before the usual time of dusk. Dusk that is premature to this extent induces a new rhabdom size that is only three-fourths of the usual night size (Williams 1982a). Furthermore, measurements were made by stimulating a cell with 500-ms flashes of light at 8-

second intervals. Laughlin et al. (1980) found that after recording sub-maximal responses to well-spaced 10-ms flashes of light from photoreceptors of the spider, Dinopis, the retina did not contain the extra photoreceptor membrane typical of a normal night state. It is possible therefore that the light given during the procedure of Horridge et al. would be sufficient to cause the dark-adapted locust cells to undergo some light-adaptive changes. No anatomical check was made to see if the retina was still in the night state after this exposure.

In locust photoreceptors, dim light elicits small discrete depolarisations, known as bumps (Scholes 1964), which represent the absorption of single photons (Lillywhite 1977). Because spontaneous bumps are extremely rare in darkness, the number of bumps is a direct measure of sensitivity (Lillywhite 1977). Thus, sensitivity can be measured under very low light intensities to ensure that no light-adaptive changes occur. The present paper explores the possible changes of photoreceptor function that are associated with the daily changes of ommatidial structure in locusts by intracellular recordings. Bump frequencies were used to determine sensitivity functions at relevant times of day. Lighting conditions throughout recording were strictly coincident with the animals' preceding day/night rhythm. Anatomical checks were made of the regions of the retina from which recordings were taken.

Materials and Methods

Apparatus

A 250-W tungsten filament lamp was run from a Hewlett

Packard HP6269 B stabilised DC power supply. Light was emitted through a narrow aperture and a 3-mm thick Schott KG 3 heat filter. It was collimated by a glass lens and could pass through one of a range of narrow-band (10-nm half-width) interference filters (413, 438, 452, 474, 494, 513, 533, 574 nm) and gelatin neutral-density filters. A second lens focused the beam on to the end of a light guide. Between this lens and the light guide, a baffle was erected to exclude stray light. A Uniblitz electronic shutter, mounted immediately in front of the tip of the light guide, controlled the duration of the stimuli. A photodiode, used to monitor its operation, showed that the minimum stimulus duration was consistently 4 ms.

Stray light was prevented from reaching the locust during recording by enclosing the preparation and recording equipment in a blackened sheet-metal Faraday cage. The light guide passed through a small sealed hole into the cage. Its stimulating end was mounted on a Cardan arm whose horizontal and vertical axes of rotation passed through the locust's eye. For recordings requiring a point source, a 0.3-mm aperture that subtended 0.1° at the eye was placed over the end of the light guide. Stimulation by a uniform extended source was achieved by placing a ground glass diffuser (Spindler and Hoyer, Göttingen), wrapped in several layers of lens tissue, in front of the eye where it subtended 40° .

Calibrations

Absolute calibration of the transmission of neutral-density and interference filters was done at frequent intervals using an International Light Inc. silicon detector (SEE100) and radiometer (IL700). For very low intensities, such as those used to elicit discrete bumps,

measurements were extended by using a photomultiplier tube (PM270D). In practice, however, one light intensity was used for as many different bump measurements as possible, so that any calibration error was avoided; the sample time was altered to obtain a significant number of bumps. The stability of the light source was periodically tested with the photomultiplier and no drift in output was detectable over 2 days; experiments usually lasted about 12 hours.

Animals

Initial experiments were performed with adult Locusta migratoria L., but when they became unavailable, adult Valanga irregularis (Walk.) were used. All animals were taken from laboratory cultures that were fed bran and wheat and maintained on a 16 h light/8 h dark cycle at 20-35°C. Initially, this cycle coincided with day and night, but later experiments were performed with "dusk" advanced to 1500 h for convenience. Light was provided by a 60 W incandescent bulb in each cage (36 x 36 x 46 cm), and two 40 W daylight-fluorescent lights shared by three cages; average illuminance in the centre of each cage was 1,000 lux. The day before experimentation, a locust was placed in the Faraday cage and surrounded with white cardboard. At night no light entered through any windows. During the day a 15 W daylight fluorescent light provided the locust with an illuminance of 150-200 lux. Temperature was $25 \pm 2^\circ\text{C}$. Humidity never varied more than 2% from a mean value between 45-55%. The animal was kept under the same conditions of temperature and humidity during preparation and experimentation.

Preparation and Recording

Prior to dusk an unanaesthetised intact locust was

mounted in a silver collar. Its head and neck were firmly waxed to the collar, which acted as an indifferent electrode as well as a support. A small hole (less than 10 facets in diameter) was cut in the dorsal cornea with a fresh chip of razor blade. It was sealed with a stiff silicone grease. Care was taken not to disrupt the optics of the eye and to keep its facets clean. After making the hole, the shape of the pseudopupil of the eye was examined for abnormalities. A change in its shape would indicate that the optics had been distorted and the eye could not be used. The locust was mounted on the stage of a micromanipulator with its prepared eye at the centre of a Cardan arm. A glass microelectrode was introduced into the eye through the hole in the cornea. The microelectrodes were filled with 3M potassium acetate and had resistances of 100-150 $M\Omega$ in the retina. Intracellular recordings of potential difference between the microelectrode and the indifferent electrode were amplified by a Grass P16 preamplifier and displayed on an oscilloscope and a chart recorder. The animal remained exposed to 150-200 lux throughout preparation.

Procedure

Although technically more difficult, it was considered that a more valid indication of the effects of different times of day on photoreceptor performance would be obtained if in each experiment recordings were made from one receptor throughout the day, rather than from different cells, which were possibly of different cell types. Attempts were made to hold each impaled cell from just before dusk until the next day, while measuring its performance at times chosen with respect to known changes of ommatidial structure (Williams 1982a). All recordings

used for analysis were from cells that showed stable resting potentials, "healthy responses", and good optics. Prior to dusk, they gave at least a 50 mV saturated response, and had a symmetrical, narrow light-adapted angular sensitivity function. Throughout the experiment, the dark-adapted resting potential never increased by more than 10 mV. A locust ommatidium has 8 photoreceptor cells, of which 6 are large and 2 small (Wilson et al. 1973). The recordings of the present report were probably all made from any of the six larger cells. A light-adapted eye (before dusk and during lights-on after "dawn") received about 150 lux from fluorescent and incandescent lighting reflected from white cardboard around the end of the light guide. Stimuli were superimposed on this background lighting. Sensitivities were determined by referring responses to an intensity/response ($V/\log I$) function obtained at the same time. Sensitivities during dark adaptation were determined directly by counting the number of bumps to an exposure of dim light. The intensity of this exposure was adjusted so that the bump rate was never more than about 3/second, and the duration of exposure ensured that a minimum of 100 bumps were recorded for each reading. In order to prevent any light adaptation, a dark-adapted cell was never exposed to light that would elicit a response greater than a train of discrete bumps.

Electron microscopy

Eyes were fixed in phosphate-buffered glutaraldehyde and paraformaldehyde, followed by OsO_4 , as described previously (Williams 1982a).

Results

All results concern the centre of the compound eye of Locusta or Valanga, so that they are comparable with a previous anatomical study (Williams 1982a). This region of the eye, which views to the side of the locust, has lower resolving power than the acute region, which samples from in front of the animal (Horridge 1978).

Ommatidial Structure

Eyes of Valanga were fixed under the conditions defined by the recording procedure; the organisation and size of the rhabdoms of Valanga and Locusta are similar (Williams 1982a). Two eyes were fixed just before dusk, and two 4-5 hours after dusk. Recordings had been made from all eyes prior to fixation, and the part of the eye examined included about 100 ommatidia in the region from which recordings had been taken. Ommatidial structure was similar to that from eyes not used for electrophysiology. No cells that may have been damaged from the recording procedure were detected. The average cross-sectional area of the rhabdom at the level of the distal nuclear region was $5 \mu\text{m}^2$ during the day and $17 \mu\text{m}^2$ at night; i.e. a 3- to 4-fold change. These measurements give quick and accurate determinations of the area of the ommatidial field stop, which is always 2.2 times greater (Williams 1982a).

In addition, two eyes of Valanga were fixed 4-5 hours after dusk, immediately after exposure to four series of 10-ms flashes ($\lambda = 560 \text{ nm}$) at 15-second intervals from an extended source. Each series of flashes began at an intensity sufficient to elicit a 35-mV response in an exposed cell, and subsequent flashes decreased in

intensity at 0.2 log-unit intervals. Rhabdoms from the exposed area of the retina appeared dark-adapted; each had a palisade of endoplasmic reticular vacuoles around it (cf. Horridge and Barnard 1965). However, they were considerably smaller than normal night rhabdoms - their cross-sectional area averaged only $7 \mu\text{m}^2$ - and pinocytotic vesicles were apparent around the bases of the microvilli.

Dark-adapted Receptor Response

After a few minutes of dark-adaptation, bumps were recorded from a locust photoreceptor in response to dim light (Fig. 1) (Scholes 1964). Bumps were very rare in complete darkness, showing that there were practically no spontaneous bumps (Fig. 1) (Lillywhite 1977). Small bumps of the kind described by Lillywhite (1978) were sometimes observed, but were ignored during analysis. Lillywhite considered them to arise from neighbouring photoreceptors that were naturally electrically coupled to the impaled cell. Although the basis of this conclusion has been questioned (Williams 1982a), it is assumed here that the small bumps at least do not originate from the impaled cell.

Spectral Sensitivity

The first experiments tested if the night and day rhabdoms had different spectral sensitivities. Measurements were taken from 5 cells. In all cases there was no detectable shift in spectral sensitivity between day and night (e.g. Fig. 2). In agreement with Lillywhite (1978), cells from Locusta were maximally sensitive to wavelengths of 474 or 494 nm. Peak wavelength of two cells from Valanga was found to be 494 or 513 nm.

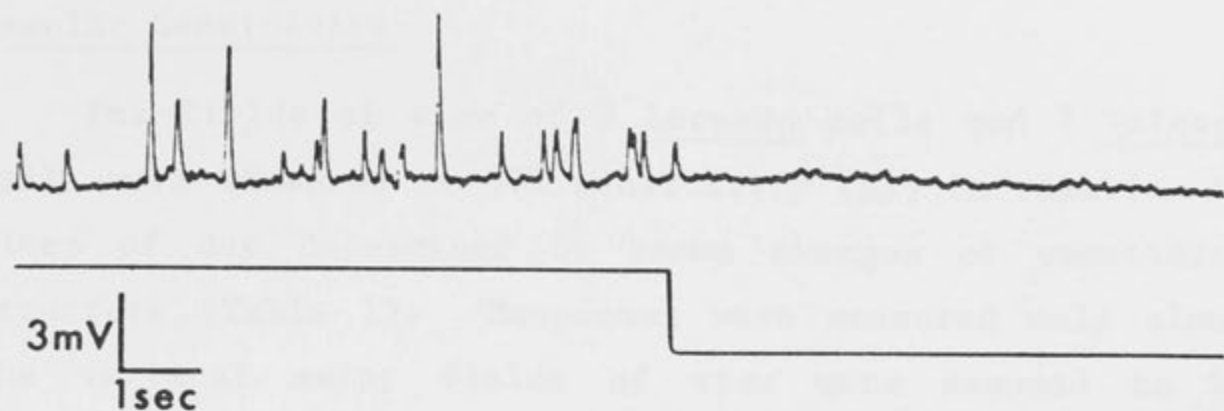


Fig. 1. Intracellular recordings of photoreceptor membrane potential at night in response to dim light. Drop in lower trace indicates when stimulus was turned off. Note the absence of bumps in darkness.

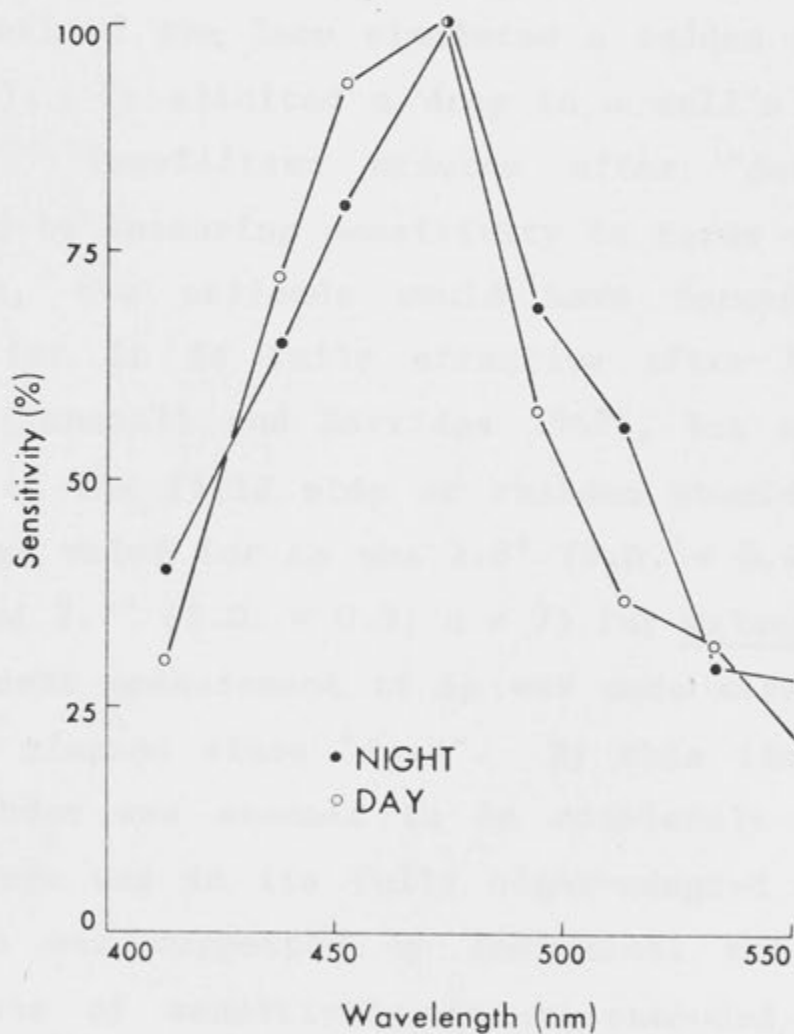


Fig. 2. Spectral sensitivity of a photoreceptor cell of *Locusta* 15 minutes after "dusk" (open circles) and 4 hours after "dusk" (closed circles); i.e., with "day" and "night" dark-adapted rhabdom states. Sensitivities to different wavelengths were measured from bump responses and normalised to a percentage of that for peak wavelength.

Angular Sensitivity

The fields of view of 3 Locusta cells and 7 Valanga cells were measured at 50% sensitivity ($\Delta\rho$) in relation to times of day determined by known changes of ommatidial structure (Table 1). Responses were measured only along the vertical axis; fields of view were assumed to be circular as shown by Wilson (1975).

Measurements of light-adapted cells were made in the process of finding their optical axes. The average value for $\Delta\rho$ was 1.9° (S.D. = 0.3° ; $n = 3$) for Locusta and 1.7° (S.D. = 0.1° ; $n = 7$) for Valanga.

Removal of the lamp simulated a sudden dusk (written as "dusk"). It elicited a drop in a cell's potential of 7-15 mV. Ten-fifteen minutes after "dusk", $\Delta\rho$ was determined by measuring sensitivity in terms of bumps. By this time, the palisade would have formed around the rhabdom, for it is fully effective after 6 minutes of darkness (Tunstall and Horridge 1967), but no changes in the size of the field stop or rhabdom should have begun. The average value for $\Delta\rho$ was 2.8° (S.D. = 0.4 ; $n = 3$) for Locusta and 2.7° (S.D. = 0.3 ; $n = 7$) for Valanga.

The next measurement of $\Delta\rho$ was made after at least 4 hours had elapsed since "dusk". By this time the larger night rhabdom was assumed to be completely assembled so that the eye was in its fully night-adapted state. This assumption was supported by anatomical monitoring, and measurements of sensitivity to an extended source (see below) which showed that sensitivity reached a plateau in less than 4 hours (Fig. 4). The average $\Delta\rho$ at night is 4.9° (S.D. = 0.3° ; $n = 3$) for Locusta and 4.7° (S.D. = 0.5° ; $n = 6$) for Valanga. The curve of the angular sensitivity function has a flat top (Fig. 3), which has not been described previously for locust photoreceptors

Table 1. Angular sensitivity measurements (in degrees) of photoreceptor cells according to time of day.

Cell ^a	Pre-	"Dusk" +		"Dawn" +					
	dusk (LA)	15m (DA)	4-8h (DA)	10m (LA)	60m (LA)	120m (LA)	140m ^b (DA)	160m ^c (LA)	9h ^d (DA)
1	1.9	2.9	5.0	2.6		1.9	3.2		
2	1.7	2.4	4.5	2.5	2.1	2.0	2.9		
3	2.2	3.1	5.1	3.6		1.9	2.5		
4	1.6	2.6	5.4	2.7	1.9	2.0	3.0	1.8	
5	1.9	2.5	4.0			2.1	2.6		
6	1.8	2.5	4.9	fixed for anatomy.					
7	1.7	3.3	4.3	fixed for anatomy.					
8	1.7	2.4	4.7	no light					
9	1.5	2.6	4.8						3.2
10	1.6	2.7	2.6	2.0					

^a Cells 1-3 from Locusta, cells 4-10 from Valanga

^b Last 20 min in darkness

^c Last 20 min in light, immediately preceded by 20 min darkness

^d Continuous darkness since night

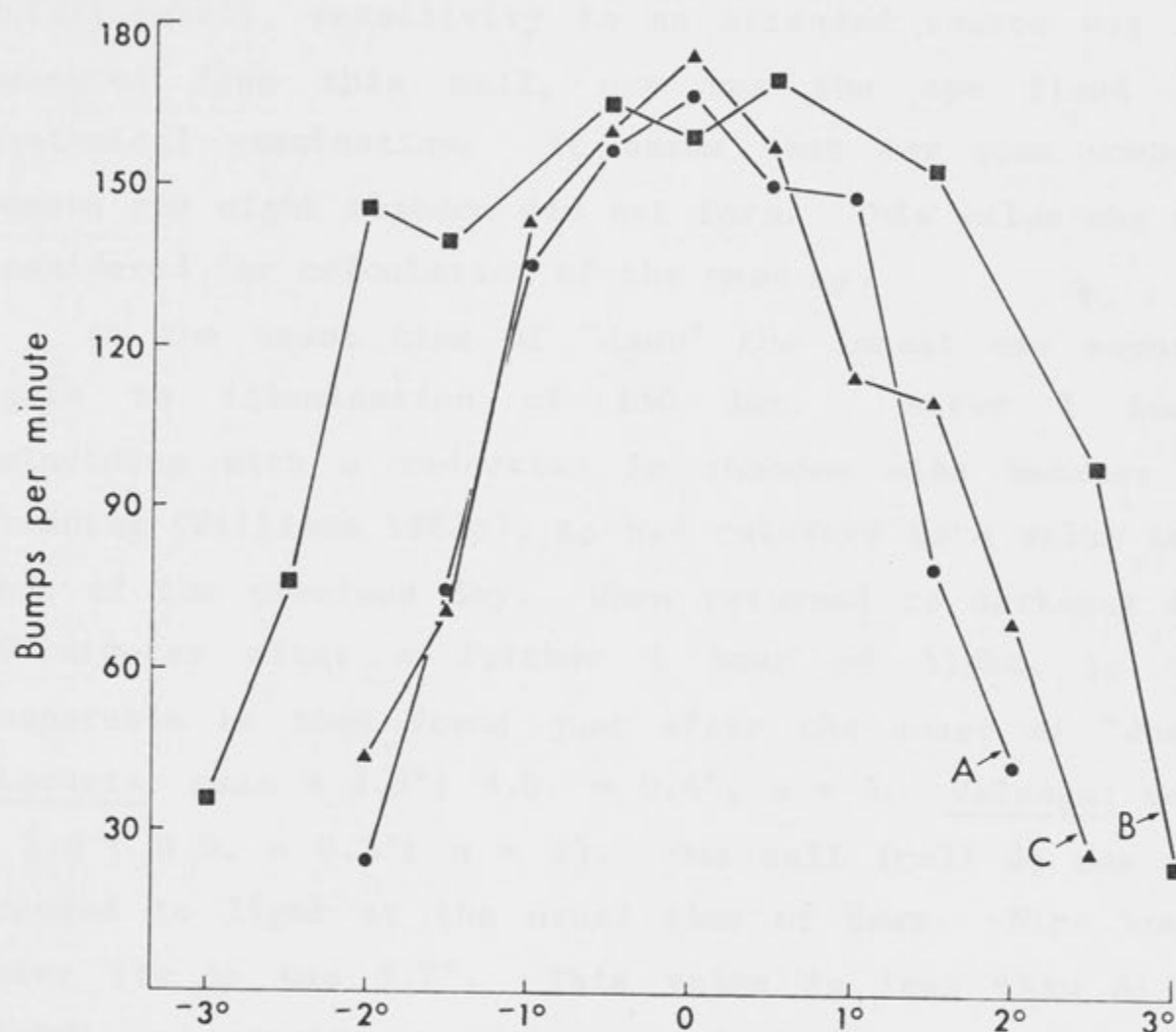


Fig. 3. Plots of the angular sensitivity function measured from a single photoreceptor (cell 1 in Table 1) just after "dusk" (A), 5 hours after "dusk" (B), and after 30 minutes dark adaptation that followed 2 hours exposure to light at dawn (C). The stimulus was a point source (subtending 0.1° at the eye) of monochromatic light ($\lambda = 413$ nm) at the one intensity for all measurements.

(cf. Tunstall and Horridge 1967; Wilson 1975; Horridge et al. 1981), or any other compound eye that is nearly diffraction limited; angular sensitivity functions of these eyes have been commonly approximated to a Gaussian function (Snyder 1977). An anomalous value for $\Delta\rho$ was measured from one cell (Cell 10 in Table 1). Unfortunately, sensitivity to an extended source was not measured from this cell, nor was the eye fixed for anatomical examination. It seems that for some unknown reason the night rhabdom did not form. This value was not considered for calculation of the mean $\Delta\rho$.

At the usual time of "dawn" the locust was exposed again to illumination of 150 lux. After 1 hour, coinciding with a reduction in rhabdom size because of shedding (Williams 1982a), $\Delta\rho$ had returned to a value near that of the previous day. When returned to darkness for 20 minutes after a further 1 hour of light, $\Delta\rho$ was comparable to that found just after the onset of "dusk" (Locusta: mean = 2.9° ; S.D. = 0.4° ; $n = 3$. Valanga: mean = 2.8° ; S.D. = 0.3° ; $n = 2$). One cell (cell 8) was not exposed to light at the usual time of dawn. Nine hours later its $\Delta\rho$ was 3.2° . This value is less than $\Delta\rho$ at night, and consistent with anatomical data, showing that shedding of photoreceptor membrane and movement of the primary pigment cells is under some degree of endogenous control (Williams 1982a).

A comparison of $\Delta\rho$ for dark-adapted cells (a) before the assembly of new photoreceptor membrane, (b) after its assembly, and (c) after the shedding of photoreceptor membrane at dawn (e.g. Fig. 3) shows that the enlarged field stop and rhabdom at night effect an increase of $\Delta\rho$ from 2.8° (overall mean; $n = 15$) to 4.9° in Locusta, and 2.7° ($n = 9$) to 4.7° in Valanga, or an increase in solid

angle, $(\Delta\rho)^2$, of 3.1-fold in both species of locust.

Absolute sensitivity

The sensitivity to a point source on the optical axis of a dark-adapted cell was independent of the different field stop and rhabdom sizes (e.g. Fig. 3). This constancy was found whether the stimulus was white light (tungsten source) or monochromatic light of wavelength, 413 nm. The latter is absorbed at only 10-40% of the efficiency of the peak wavelength (Lillywhite 1978; present results, e.g. Fig. 2), so that its use provides a more sensitive test for any change in absorption efficiency of the rhabdom. We already know that a very high proportion of peak wavelength light incident on the rhabdom is absorbed (Lillywhite 1977). If a means to change absolute sensitivity between the day and night states existed, it would be more likely to be detected from a change in the absorption of an off-peak wavelength.

Sensitivity to an Extended Source

Sensitivity to an extended source, subtending 40° at the eye and therefore illuminating about 400 ommatidia (based on the interommatidial angle measurements of Burtt and Catton (1954)), was monitored throughout the night from 4 cells (Fig. 4) as soon as bumps were distinctive after "dusk"; the first measurement was usually completed by 3-5 minutes after the onset of darkness. For the first 1-3 hours, an increase in sensitivity of about 1 log unit was detected. However, it was not feasible to determine a baseline sensitivity, for sensitivity should have begun to increase prior to the first measurement. The photomechanical changes that produce the palisade probably begin immediately after the onset of darkness. An

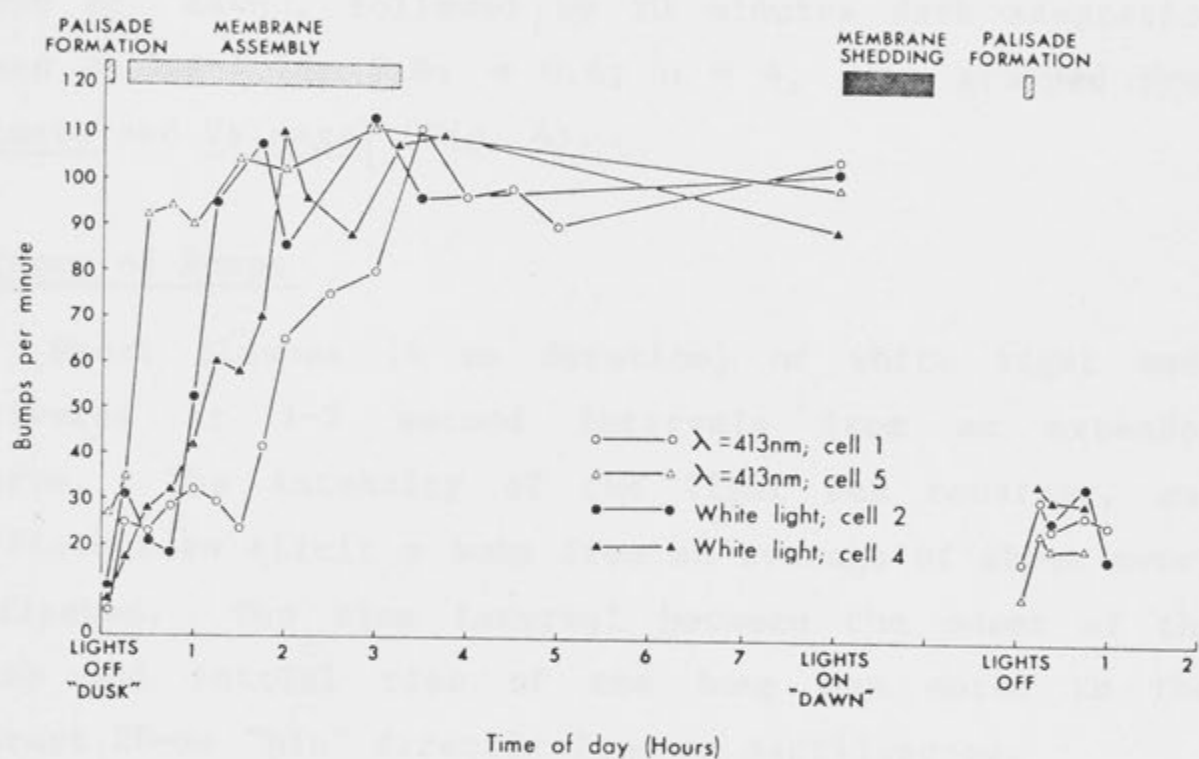


Fig. 4. Sensitivity of single photoreceptors to an extended source. All recordings, except for those obtained during the first 15 minutes of darkness, were made with one light intensity, which was chosen so that the maximal response rate would be about 100 bumps/minute. The sensitivities shown have been normalised so that the mean sensitivity of the fully night-adapted state is exactly 100 bumps/minute.

optically equivalent effect, achieved by moving pigment granules within the photoreceptor cells and known as the longitudinal pupil mechanism, is completed within 10-60 seconds in many insects (Stavenga 1979). In 2 out of the 4 cells, sensitivity noticeably plateaued for a short period subsequent to the second measurement, made 15 minutes after "dusk" (Fig. 4). The magnitude of the rise that followed this plateau averaged 3.9-fold, and was comparable to the drop in sensitivity found after 2 hours light at "dawn", followed by 20 minutes dark adaptation (mean = 3.8-fold; S.D. = 0.6; $n = 4$, data grouped from Locusta and Valanga) (Fig. 4).

Latency of Bumps

Short flashes (4 ms duration) of white light were delivered at 1-2 second intervals from an extended source. The intensity of the light was constant, and sufficient to elicit a bump from an average of about every 2 flashes. The time interval between the onset of the flash and initial rise of the bump was noted to the nearest 20-ms "bin" directly from an oscilloscope.

The latency was measured from 4 dark-adapted cells. In all cells, no difference was apparent in the bump latency between the day and night rhabdom states (e.g. Fig. 5).

Discussion

Spectral Sensitivity

The spectral sensitivity functions of cells were unchanged between day and night, and were similar to those found by Lillywhite (1978). Because the spectral sensitivity is constant, no adjustment of the angular and

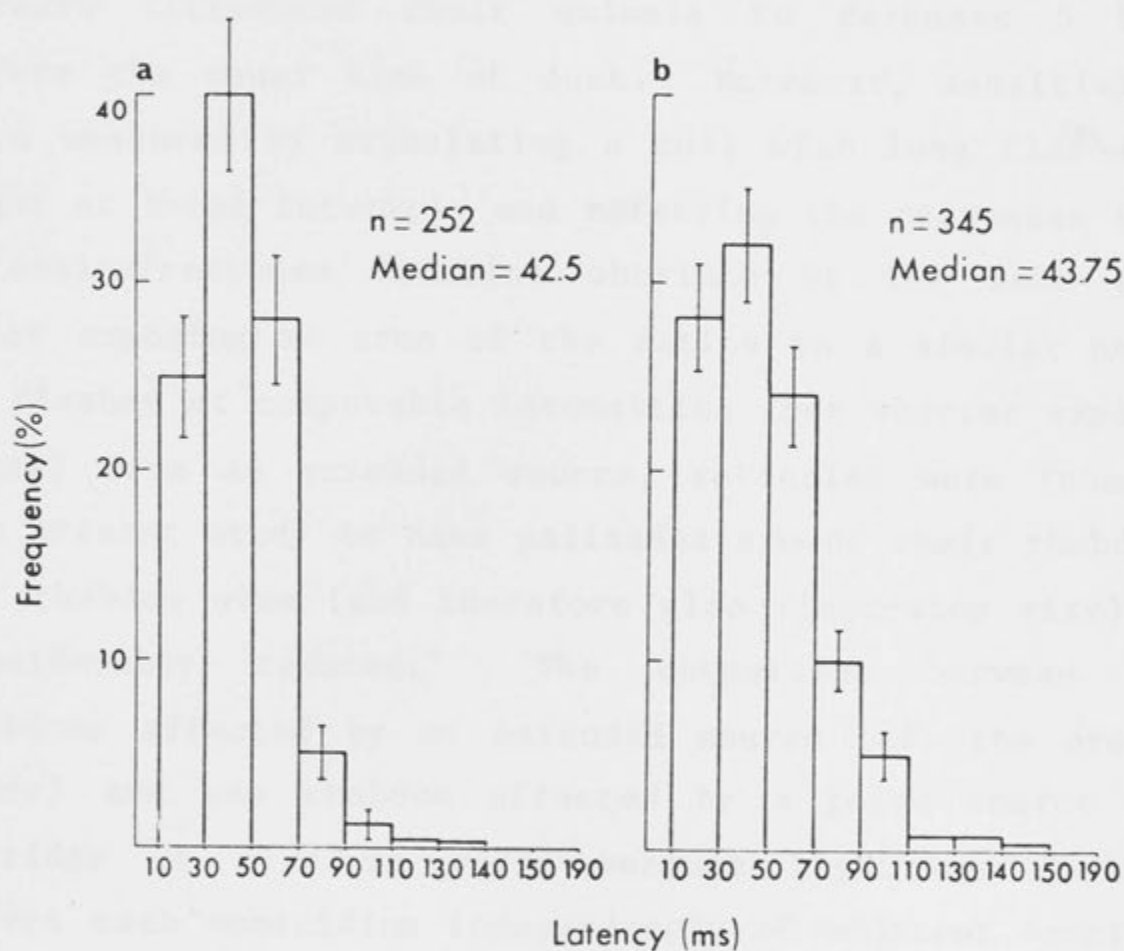


Fig. 5. Histogram of bump latencies of a single cell of *Locusta* recorded (a) 10-15 minutes after "dusk" and (b) 5 hours after "dusk". Error bars extend 1 S.D. Temperature was 25°C at both times.

absolute sensitivities was required in order to make comparisons between the day and night states.

Angular Sensitivity

Horridge et al. (1981) report dark-adapted angular sensitivity measurements of day (1.25° – 2.0°) and night (up to twice as large an angle) rhabdoms in the acute region of the compound eye of the Valanga. However, these workers introduced their animals to darkness 5 hours before the usual time of dusk. Moreover, sensitivities were measured by stimulating a cell with long flashes of light at brief intervals and referring the responses to an intensity/response function obtained at the same time. After exposing an area of the retina to a similar number of flashes at comparable intensities (but shorter exposure times) from an extended source, retinulae were found in the present study to have palisades around their rhabdoms, but rhabdom size (and therefore also field-stop size) was considerably reduced. The comparison between many rhabdoms affected by an extended source (cf. the present study) and one rhabdom affected by a point source (cf. Horridge et al.) is valid because light and darkness affect each ommatidium independently of adjacent ommatidia (Williams 1982b).

In an attempt to maintain cells in their natural light condition, the night-adapted eyes in the present experiments were exposed only to dim light which elicited trains of discrete bumps. Electron microscopy showed that this exposure was not sufficient to affect the structure of the night rhabdom. The values of $\Delta\rho$ obtained from the night-state eye of Valanga in the present experiments (mean = 4.7°) are larger than the values of Horridge et al. partly because they are from a less acute region of

the eye. Part of the reason also appears to be that they were taken from a truer night state.

Still wider acceptance angles were found in Locusta by Tunstall and Horridge (1967). They measured 6.6° for dark-adapted cells and 3.4° for light-adapted cells. However, such wide angles were probably a result of optical damage inflicted during preparation of the eye. Wilson (1975) took great care to preserve the optics and measured 2.5° and 1.4° for dark- and light-adapted cells respectively. In this study, before recordings were made, eyes were dark-adapted for 30 minutes, then light-adapted, dark-adapted for a further 20 minutes, and finally light-adapted again by flashes during alignment of the cell. Measurements obtained under these conditions should probably be compared to those from the dark-adapted day-state eyes (just after "dusk" or dark-adapted after light at "dawn") of the present study. Wilson's values for $\Delta\rho$ are then slightly less than those presented here (Table 1), but not inconsistent with them. This difference, although possibly not significant, could result from several factors. Wilson does not specify the region of the eye he examined; he may have recorded from a more acute region. Moreover, his dark-adapted measurements were obtained from responses to 70-ms flashes of light, 8-10 seconds apart, thus possibly inducing some light-adaptive changes that were avoided in the present experiments. Lastly, the background illumination used by Wilson to light adapt cells was considerably stronger than that used in the present study, so that a greater degree of light adaptation may be responsible for his narrower light-adapted value for $\Delta\rho$. Like Wilson's results, those of the present study are considered to be unaffected by optical damage.

The advantage of increasing $\Delta\rho$ when ambient luminance decreases has been previously discussed in general terms (review: Snyder 1979). Briefly, it provides a means of increasing the number of captured photons at a time when a scarcity of photons lowers the signal-to-noise ratio of the photoreceptors. However, the increase of $\Delta\rho$ "trades-off" with a decrease in spatial acuity. From this point of view it is instructive to compare $\Delta\rho$ with the interommatidial angle ($\Delta\phi$).

For two points to be separately resolved, they can be no closer together than $2 \Delta\phi$, so that one ommatidium corresponds to each point and a central ommatidium detects the space between. In this limiting situation $\Delta\rho$ must be sufficiently less than $2 \Delta\phi$ for the central ommatidium to receive less than 19% of the maximum intensity of one point (Rayleigh criterion); otherwise the two points blur into one (cf. Fig. 23 in Laughlin 1980). In some animals $\Delta\rho$ is very much less than $2 \Delta\phi$; for example, $\Delta\rho$ is approximately equal to $\Delta\phi$ over the whole light-adapted mantis eye (Rossel 1979). Such under-sampling is argued to increase contrast resolution, or permit a high spatial acuity with fewer ommatidia by scanning (cf. discussions by Laughlin 1980, Kirschfeld 1982).

The locust eye has an oval shape. Using the pseudopupil of the eye, Burtt and Catton (1954) calculated $\Delta\phi$ along the vertical axis to be 1.1° , and 2.4° on the horizontal axis in the central region of the eye of Locusta. Measured values of $\Delta\rho$ are compared to these values of $\Delta\phi$ in Fig. 6. During the day $\Delta\rho$ is lowered so that $\Delta\rho/\Delta\phi$ equals 1.7 and 0.8 along the vertical and horizontal axes respectively, thus providing increased

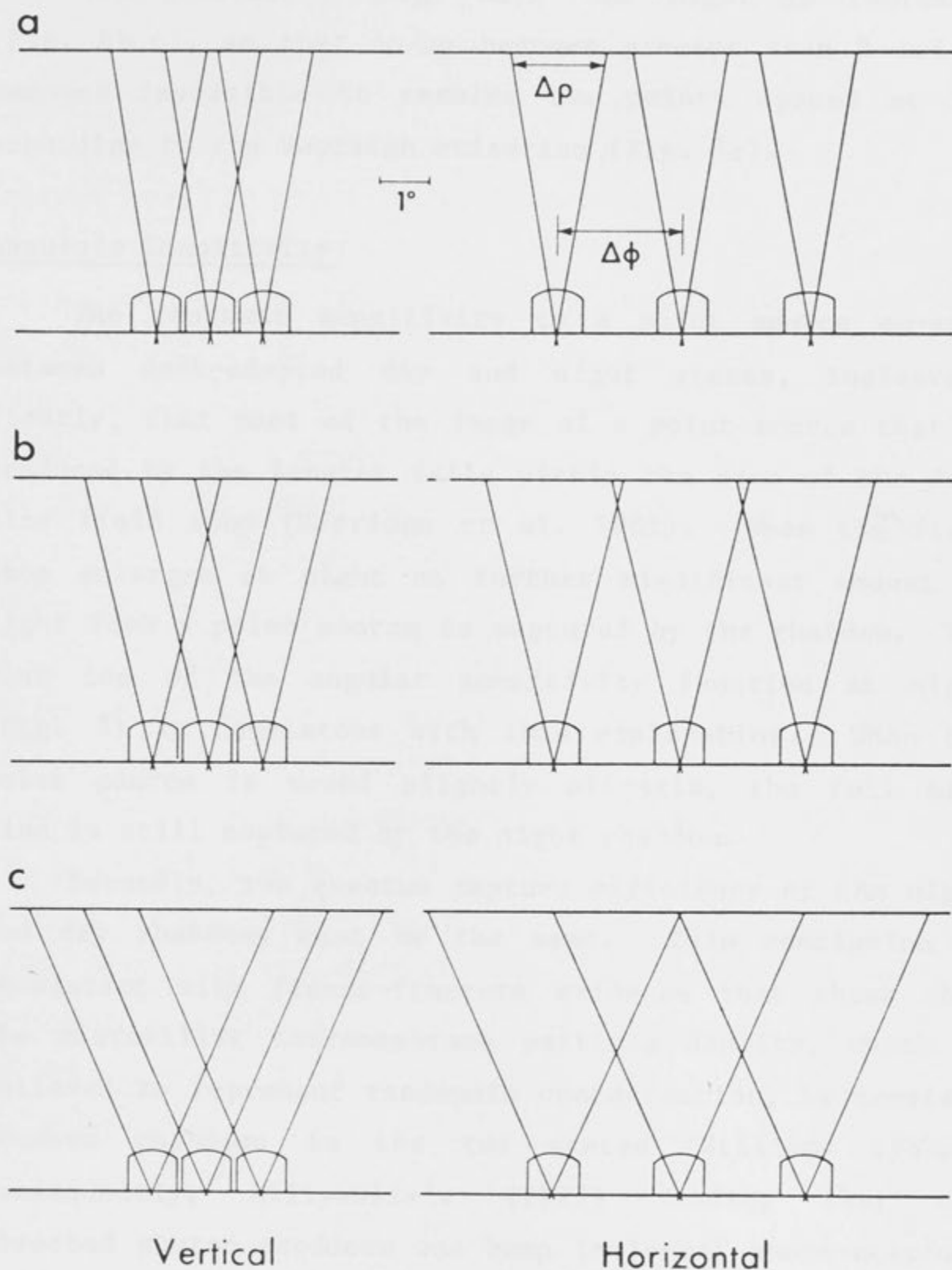


Fig. 6. Schematic representation of the spatial acuity of the eye of *Locusta* in its different adaptive states. Angular measure has been transformed to linear measure, however the diagram is designed such that the measured fields of view ($\Delta\rho$) are shown at a magnification of 10 times. Interommatidial angles ($\Delta\phi$) in the vertical and horizontal axes of the eye are taken from Burtt and Catton (1965). The values for $\Delta\rho$ are the means found in the present study: a light-adapted during the day; b 15

contrast resolution (Fig. 6a). At night $\Delta\rho$ increases (Fig. 6b,c), so that $\Delta\rho/\Delta\phi$ becomes greater than 2 and it becomes impossible to resolve two points spaced at $2\Delta\phi$ according to the Rayleigh criterion (Fig. 6c).

Absolute Sensitivity

The constant sensitivity to a point source on-axis between dark-adapted day and night states, indicates, firstly, that most of the image of a point source that is produced by the lenslet falls within the area of the day-size field stop (Horridge et al. 1981). When the field stop enlarges at night no further significant amount of light from a point source is captured by the rhabdom. The flat top of the angular sensitivity function at night (Fig. 3) is consistent with this explanation. When the point source is moved slightly off-axis, the full Airy disc is still captured by the night rhabdom.

Secondly, the quantum capture efficiency of the night and day rhabdoms must be the same. This conclusion is consistent with freeze-fracture evidence that shows that the microvillar intramembrane particle density, which is believed to represent rhodopsin concentration, is constant between rhabdoms in the two states (Williams 1982a). Consequently, Lillywhite's (1977) finding that one absorbed photon produces one bump in locust photoreceptors (that were dark-adapted for "at least 2 hours", and therefore probably possessed a newly assembled "night" rhabdom: cf. discussion by Williams 1982a) can be extended to include rhabdoms of both the day and night states.

Sensitivity to an Extended Source

The effective gain in the number of photons captured by the larger night rhabdom and field stop is shown by the

photoreceptors' increase in sensitivity to a uniform extended source subsequent to the first 15 minutes of darkness after "dusk"; that is, after the palisade is fully formed (Tunstall and Horridge 1967). Because the distal end of the locust rhabdom remains at a set distance from the lenslet (Williams 1982a), and the absorption efficiency of the rhabdom is constant (above), this gain is directly attributable to, and should be of the same magnitude as the increase in cross-sectional area of the field stop. The extent of changes in cross-sectional area of the field stop is similar in Locusta and Valanga (Williams 1982a), so that data from the two locusts have been grouped.

The cross-sectional area of the field stop was found (from measurements of rhabdomal cross-sectional area) to increase by 3- to 4-fold between day and night. This gain is consonant with the measured increase in sensitivity to an extended source: the night rhabdom was 3.8 times (overall mean; S.D. = 0.5; $n = 6$), or 0.6 log units, more sensitive than the dark-adapted day rhabdom. Previously, a 4.7-fold difference in the cross-sectional areas of the rhabdom and field stop was found between day and night (Williams 1982a), but in that study illumination during the day was 1 log unit higher than in the present study, and the day-state rhabdom and field stop were slightly smaller: at the level of the distal nuclear region, the cross-sectional area of the rhabdom was $3.6 \mu\text{m}^2$, compared to $5 \mu\text{m}^2$ in the present study. (It seems that as in the mosquito (White and Lord 1975), rhabdom size in the locust is modulated by intermediate light intensities.)

During the assembly of new photoreceptor membrane, rhabdomeres appear disordered in electron micrographs (Williams 1982a; Stowe 1980b for a similar event in a

crab). Although it is not known if a whole rhabdomere is in disarray at a given time, it appears that segments up to 100 μm long are disordered for up to 20-30 minutes. It was therefore hypothesised that the disarray might affect photoreceptor sensitivity. However, no evidence of such an effect was apparent from regular sampling of sensitivity to an extended source of white light or monochromatic light of an off-peak wavelength during the assembly period (cf. Fig. 4). No significant drop in sensitivity was apparent at any time between "dusk" and "dawn", and the only disturbance (found in 2 out of 4 cells) in an otherwise steady rise to maximal sensitivity from "dusk" can be assumed to have occurred after the completion of the palisade and before the rhabdom and field stop had begun to enlarge. As for the test of constancy of absorption efficiency, use of an off-peak wavelength (413 nm) provided a more sensitive test than using white light. Because of the exponential nature of light absorption down a receptive segment, a substantial proportion of an efficiently-absorbed wavelength will be absorbed by a small proportion of the rhabdom, usually the distal part. Absorption of an off-peak wavelength is distributed more evenly down the rhabdom, so that abnormal functioning of a segment of the rhabdom should be more readily apparent. This negative result poses the question of whether or not the disarray is an artefact of electron microscopical preparation. Alternatively, and more likely, the disarray may simply not affect absorption efficiency. After a damaging exposure to incandescent lighting, the ERG sensitivity of rat eyes returns to normal well before the outer segment discs recover their regularity (Kuwabara 1970). If a parameter of sensitivity is affected, then it may be polarisation sensitivity,

which is, in any case, quite low in locusts (Shaw 1969; Lillywhite 1978; own observations).

Latency of Bumps

The distribution of bump latency found in the present study was similar to that found by Lillywhite (1977) and, in a more detailed analysis, Howard (1982). That it was also found to be the same in both rhabdom states has some relevance to current ideas about phototransduction. Phototransduction is considered to involve absorption of light, followed by a local release of an unknown transmitter substance, which interacts with receptors in the membrane that control sodium conductance channels. The microvilli are twice as long at night (Williams 1982a). Therefore, the present results indicate that if the conductance channels are restricted to the bases of the microvilli, as suggested by Hamdorf and Kirschfeld (1980), any time taken for the transmitter to diffuse down the microvillus to the channels is not a major part of the phototransductive latency. Alternatively, of course, if the diffusion time is significant, then the similar latencies found for short and long microvilli indicate that conductance channels must be present along the length of the microvilli.

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CONCLUDING REMARKS

This thesis has described aspects of the mechanisms, functional effects, and control of photoreceptor membrane turnover in insect compound eyes. In conclusion, the current status of these topics in all eyes is briefly discussed.

Mechanisms of Turnover

Chapter V showed that in the open rhabdom of a tipulid fly receptor membrane was shed primarily via an extracellular route rather than by endocytosis from the bases of the microvilli, which is more common among compound eyes. A similar route was also apparent in the open rhabdom of the blowfly (chapter VI). Different retinal organisations are perhaps compelled to have different strategies for disposing of membrane. Among the invertebrates the potential for different strategies should be great, for, as Eakin (1972) notes, from the viewpoint of variation in retinal design the vertebrates are "dull and uninteresting" in comparison with the invertebrates. Photoreceptive microvilli that project into the lumen of seastar ocelli shed by endocytosis into the sensory cell, and by abscission into the ocellar lumen, from where they are phagocytosed by supportive and corneal cells (Eakin and Brandenburger 1979; Brandenburger and Eakin 1980). Microvilli of leech photoreceptors appear to shed into a central vacuole (or phaosome) which they surround (Walz 1979). Recently, Waterman and Piekos (1981) proposed that wandering hemocytes might phagocytose some photoreceptor membrane that is shed during turnover in crayfish retinae. In the third eye of lower vertebrates, macrophages that wander in the lumen of the

eye are thought to phagocytose shed outer segment discs (Eakin 1973). Ameboid phagocytes also appear to assist the retinal pigment epithelium in the uptake of shed outer segment discs in the lateral eyes of fish (O'Day and Young 1978; Braekevelt 1980). In this role they are perhaps particularly important in the retinae of deep-sea fish and some eels, whose photoreceptors are tiered so that there are several layers of outer segments that do not abut the pigment epithelium (Locket 1977; Shapley and Gordon 1980).

In addition to membrane turnover in bulk, as demonstrated by microscopists, it appears that membranes can also have individual molecules replaced without requiring disruption and reassembly of their structure (review: Holtzman et al. 1979). This conclusion arises from the heterogeneity of turnover rates found by biochemists for different molecules. For example, the lipids of rod outer segments appear to turn over faster than the proteins (Bibb and Young 1974), indicating that they may undergo exchange with lipids of other rod cellular organelles (Anderson et al. 1980). They also appear to be actively metabolised within the outer segments (Anderson et al. 1980). Many authors feel that what is needed from future studies is some idea of the relative importance of the biochemists' molecule-by-molecule processes and the microscopists' "bulk" processes under varying conditions (e.g. Holtzman et al. 1979, regarding membranes in general).

Functional Effects of Turnover

Changes in photoreceptor function that are related to photoreceptor membrane turnover through a variation of rhabdom size were considered and investigated in this thesis. However, this modulation of photoreceptor

performance is probably a secondary effect of turnover. The generally accepted primary reason for why photoreceptor membrane, like all other membranes, turns over is that the membrane in some sense "ages" (Young 1976; Blest 1978, 1980). Because photoreceptor cells per se are not replaced during the life of the animal, their cellular components must turnover if the cells are to maintain functional integrity. The means to test this hypothesis will arise when studies on the control of turnover enable the processes of turnover to be manipulated. For example, if photoreceptor membrane were prevented from turning over, would it become less efficient at transducing light?

Control of Turnover

We now know that photoreceptor membrane turnover can be influenced by ambient lighting and endogenous factors (in many cases both: discussion in chapter VII), and that the effects of ambient lighting are exerted locally (chapter VIII). The next steps of investigation should clearly attempt further dissection of the control mechanisms: How are the effects of light and darkness mediated? What is the nature of the endogenous factors?

As suggested in chapter VIII, a likely candidate for the mediator of the effects of ambient lighting is intracellular Ca^{++} . Its concentration varies with light and darkness, and is responsible for changes in photoreceptor sensitivity and receptor cell pigment migration (references in chapter VIII). In vitro eye preparations, as developed for Xenopus (Flannery and Fisher 1979; Besharse et al. 1980), and a locust (Payne 1981) and crab (Stowe 1982), should be useful for tests of this hypothesis.

The basic mechanisms leading to circadian oscillations in general are still unknown; current research employs a wide variety of biochemical models, and mathematical and control theory descriptions (Engelmann and Schrempf 1980). Consequently, the mechanisms of endogenous control of photoreceptor membrane turnover are obscure. Some characteristics of the mechanisms have been identified, but findings vary among different animals. Efferent input is essential for the circadian rhythmicity of turnover in Limulus (Chamberlain and Barlow 1979), but endogenous initiation of photoreceptor membrane assembly in the crab, Leptograpsus, proceeds normally in an isolated retina (Stowe 1982). A model for the circadian control of shedding in the rat involves a synchroniser in the central nervous system, and an autonomous oscillator in each retina. Synchronisation of each oscillator is dependent on an intact optic nerve (Tierstein et al. 1980). One consistent feature emerging is that turnover does not appear to be controlled by systemic elements (cf. Stowe 1982; LaVail and Ward 1978; Tamai et al. 1978). Consequently, in vitro eye preparations will probably become of paramount importance in investigations of the mediation of the effects of both ambient lighting and the endogenous control.

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APPENDIX

THE LOCAL DELETION OF A MICROVILLAR CYTOSKELETON FROM PHOTORECEPTORS OF TIPULID FLIES DURING MEMBRANE TURNOVER

A.D. Blest, S. Stowe, W. Eddey, D.S. Williams

Abstract. The distal regions of the photoreceptor microvilli of tipulid flies are shed to extracellular space during membrane turnover. Prior to abscission, the microvillar tips undergo a transformation: they become deformed, and after conventional fixation for electron microscopy are relatively electron lucent compared to the stable, basal microvillar segments. We now show that the electron lucent segment is an empty bag of membrane whose P-face after freeze-etch preparation appears as densely particulate as the remainder of the microvillus. Transformation is achieved by the local deletion of a microvillar cytoskeleton which consists of a single, axial filament linked to the plasma membrane by side-arms. The filament may be partially preserved by the chelation of Ca^{2+} ; the provision of a divalent cation (Mg^{2+} or Ba^{2+}) stabilises the side-arms during subsequent fixation, as has been shown previously for the rhabdomeral cytoskeleton of blowflies. Incubation of the isolated retina in the presence of 0.25 mM Ca^{2+} at room temperature for 10-20 min causes proteolysis of the cytoskeleton which is blocked by as little as 0.5 mM of the Ca^{2+} -activated thiol protease inhibitors Ep-475 or Ep-459. Loss of the cytoskeleton is accompanied by deformation of all regions of the microvilli. Local deletion of the cytoskeleton from the transformed zone of the normal rhabdom is sufficient to explain deformation of the microvillar tips, but not their subsequent abscission. The intimate association between a Ca^{2+} -activated thiol protease and the cytoskeleton implied

by the great rapidity of proteolysis calls for a reassessment of published studies of membrane turnover by autoradiography, and the nature of light-induced damage to arthropod photoreceptor membranes.

INTRODUCTION

Photoreceptors of the compound eyes of tipulid flies shed rhabdomeral membrane to extracellular space during the catabolic phase of turnover. In each rhabdomere of an ommatidium, the tips of the microvilli are transformed so as to appear relatively electron lucent in thin sections. At a later stage the microvillar segments forming the transformed zone become swollen and distorted (figures 1 and 2); they are later shed by abscission. Shed membrane is rapidly removed from the extracellular space within the ommatidium and returned by phagocytosis to the receptors, in which it accumulates as large, secondary lysosomes. In Ptilogyna, shedding occurs during the periods after dawn and is followed by a slow, renewed transformation of the distal regions of the microvilli which defines the zone that will be shed some 24 h later (Williams & Blest 1980). These authors and Blest (1980) considered that the loss of electron density by the transformed region might represent the withdrawal of a stabilising cytoskeleton from the microvillus tips.

Blest et al. (1982) have shown that the rhabdomeral microvilli of blowflies contain a labile, Ca^{2+} -dependent cytoskeleton which is largely destroyed by conventional methods of fixation. A single, axial filament is linked to the plasma membrane by side-arms. In addition, microvilli are bonded together by external bridges which were demonstrated both in thin sections and in freeze-etch

replicas. The mechanical importance of the cytoskeleton is not yet understood, but it is reasonable to suppose that it plays some part in generating the remarkable regularity of the microvillar architecture (Horridge & Blest 1980; Stowe 1980).

In the present paper we show that the microvillus tip is transformed by the removal of its contents, and that the deleted components exhibit many of the features of the cytoskeleton found in blowfly rhabdoms. The organisation of the integral membrane proteins of the microvillus, insofar as it can be resolved by the freeze-etch technique, remains the same in both the transformed and non-transformed regions.

MATERIALS AND METHODS

The large tipulid fly Ptilogyna (Plusiomya) spectabilis (Skuse) and various species of a genus of smaller tipulids, Leptotarsus, were collected in the field at sites around Canberra. Several species of Leptotarsus commonly co-exist in a given Australian habitat, and it was impracticable to identify the subjects of experiments to species. Flies were stored for 1-4 days under a natural environmental light cycle as described by Williams (1980a) and Williams & Blest (1980), or for some experiments in continuous darkness.

Fixation for transmission electron microscopy

Flies were decapitated, the heads immediately divided into two halves, and dissection continued beneath various modifications of a basic stabilising buffer (20 mM imidazole, 150 mM NaCl, 4 mM KCl, 15 mM MgCl₂, 80 mM glucose, 10 mM ethyleneglycol-bis-(β -amino-ethyl ether)

N,N'-tetra-acetic acid (EGTA), 1 mM phenylmethanesulphonyl fluoride (PMSF), 0.5% dimethyl sulphoxide (DMSO), pH 6.8-7.0). Head structures, including air sacs, were trimmed away until only the laminae of the optic lobes remained attached to the retinae. After treatment with stabilising buffer for from 10-20 m at room temperature, retinae were fixed by means of the following sequence of treatments:

(i) 2% paraformaldehyde, 2.5% glutaraldehyde, 15 mM MgCl_2 , 10 mM EGTA, 0.09 M sodium cacodylate, 0.12 M sucrose, pH 6.8, at room temperature for 30 m. Cut each retina into two to three pieces.

(ii) Wash once, and then fix for a further 60-90 m in the same cacodylate-buffered aldehyde solution with MgCl_2 and EGTA replaced by 10 mM ethylenediaminetetra-acetic acid disodium salt (EDTA).

(iii) Transfer to 0.4% low molecular weight tannic acid (LMWT: Simionescu & Simionescu 1976) in cacodylate buffer with 4 mM EDTA for 2-4 h at room temperature followed by overnight at 4°C, or for 10-12 h at room temperature.

(iv) Wash in cacodylate buffer with 10 mM EDTA at room temperature for 1-3 h.

(v) Post-fix in 1% OsO_4 in cacodylate buffer for 1 h.

(vi) Wash in distilled water for 30 m, dehydrate through an ethanol series and propylene oxide and embed in Araldite.

With the exceptions noted below, Ca^{2+} must be chelated from all media used in preparing rhabdoms for electron microscopy if the cytoskeleton is to survive (Blest et al. 1982). In addition, divalent cations, preferably Mg^{2+} must be present to conserve the side-arms. However, Williams (1980b) demonstrated that the presence of Ca^{2+} during fixation caused the conversion of microvillar membranes to myelin figures, and White &

Michaud (1981) found that other divalent cations, including Mg^{2+} produce the same effects. The schedule of fixation given above floods the tissues with Mg^{2+} for a period sufficient to ensure the cross-linking of side-arm proteins by glutaraldehyde, and then removes it with EDTA before the stage of osmication during which myelin figures are produced.

A number of modifications to the basic protocol are described under Results. In outline, (a) hypotonic stabilisation buffers were employed in which the concentrations of NaCl and glucose were lowered to 50 mM and 40 mM respectively; (b) the protease inhibitor N- α -p-tosyl-L-lysine chloromethyl ketone (TLCK) was used in combination with PMSF, both at concentrations of 1 mM; (c) the powerful and specific inhibitor of thiol proteases Ep-475 (E-64c) and its analogue Ep-459 (Hanada et al. 1978; Tamai et al. 1981) were used at 0.5-1 mM in modifications of the stabilisation buffer. These reagents irreversibly inhibit the Ca^{2+} -activated neutral thiol proteases associated with vertebrate neurofilaments (Kamakura et al. 1981). (d) In a few experiments, $BaCl_2$ was substituted for $MgCl_2$ in the buffer.

Observations on thin sections and replicas

Grey sections were stained with uranyl acetate and lead citrate after mounting on formvar coated 400-mesh grids, and viewed in an Hitachi H600 electron microscope. The relationship between section thickness and microvillus diameter, which are of the same order of magnitude, produces some problems of interpretation. These, and the difficulties of matching features of freeze-etch replicas to those seen in sections of rhabdoms are discussed by Blest et al. (1982).

Preparation of freeze-etch replicas

Retinae were either fixed without pre-treatment, or after dissection beneath the basic stabilisation buffer. Following the second stage of fixation, glycerol was added to the EDTA-containing fixative to a final concentration of 25-30%. Retinae mounted on gold holders were frozen in Freon-22, cooled by liquid nitrogen, fractured at -110 to -118°C and 10^{-7} to 10^{-8} mbar in a Balzers BAF 300 apparatus equipped with a quartz crystal thin film monitor, and coated immediately with platinum/carbon and carbon films. The replicas were cleaned with chromic acid and/or chromosulphuric acid, mounted on 100-mesh grids coated with formvar, and viewed in an Hitachi H500 electron microscope. When necessary, replicas were removed from their grids and recleaned, using the procedure described by Stowe (1981).

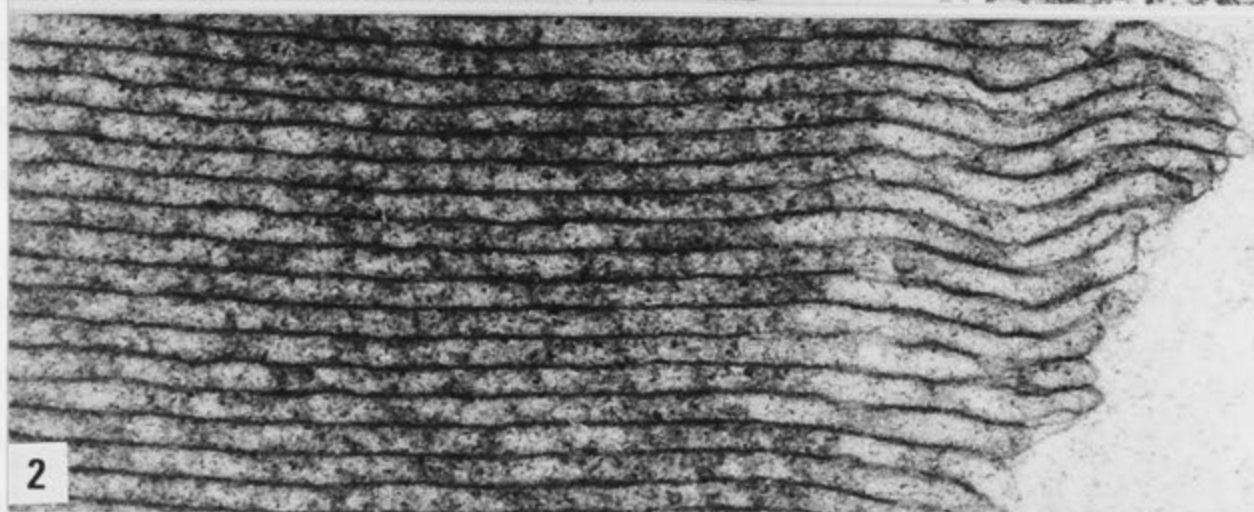
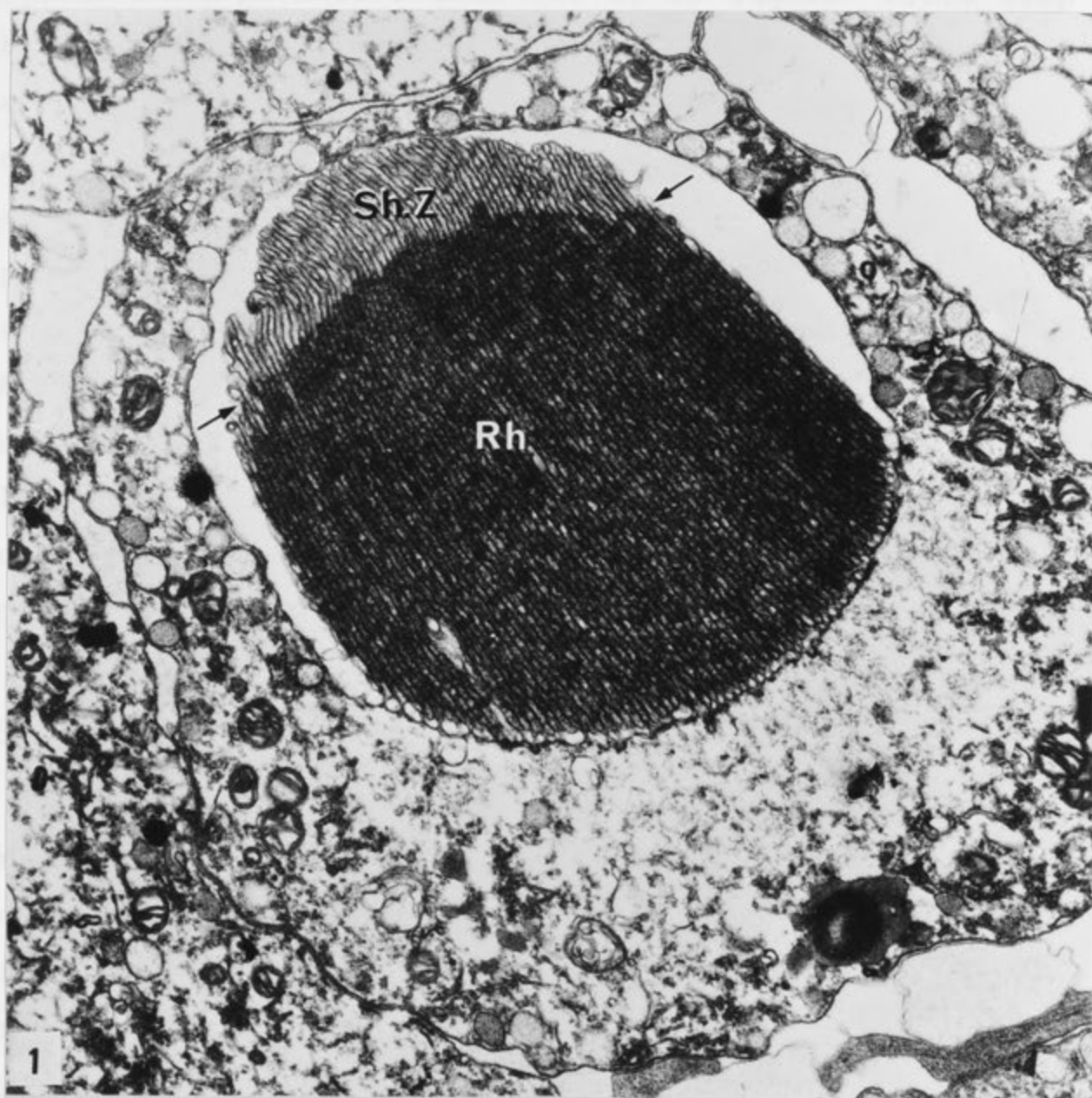
RESULTS

Anatomy

The retinal anatomy of Leptotarsus resembles that of Ptilogyna, described in detail by Williams (1980a, 1981). Each ommatidium consists of six peripheral receptors (R1-6) arranged in a circle, and two central, tiered receptors (R7,8). Distally the rhabdoms of R1-6 and R7 are contiguous; in the mid-region of the ommatidium all rhabdomeres are separated by a shared, extracellular space; proximally, R1-6 and R8 are each invaginated to form a pocket which encloses the rhabdom and the extracellular space surrounding it (e.g. figure 1). Although each receptor contains screening pigment, most abundantly in the proximal, pocketed region, it does

Fig. 1. (over page) Transverse section through a rhabdom of Leptotarsus lying in the proximal pocket formed from its own photoreceptor, after pretreatment with the basic stabilisation buffer, and post-fixation mordanting in LMWT. The shedding zone (Sh.Z) is clearly delimited (arrows) from the rest of the rhabdom (Rh) by its lesser electron density. X 16,200.

Fig. 2. (over page) Longitudinal section through rhabdomeral microvilli of Leptotarsus after conventional fixation. The microvillar lumen shows little evidence of organised structures, and the shedding zone at the right of the figure is composed of deformed microvillar tips of lesser electron density than the stable region to the left. X 54,000.



not act as a longitudinal pupil (cf. Kirschfeld & Franceschini 1969). The primary pigment cells just beneath the crystalline cone act as an annular field stop that opens to admit light to R1-6 in the dark-adapted state at night and constricts during the day largely to confine incoming light to the two central, tiered rhabdoms. The prefixation treatments used in the present experiments chelate Ca^{2+} , and thus effectively force the receptors into the dark-adapted state (Kirschfeld & Vogt 1980), irrespective of the illumination under which dissection is carried out. The rhabdoms of tipulids are only some 30-50 μm in length (Williams 1980a). Microvillar diameters may vary between 50 to over 60 nm, occasionally within a single rhabdom.

The shedding zone

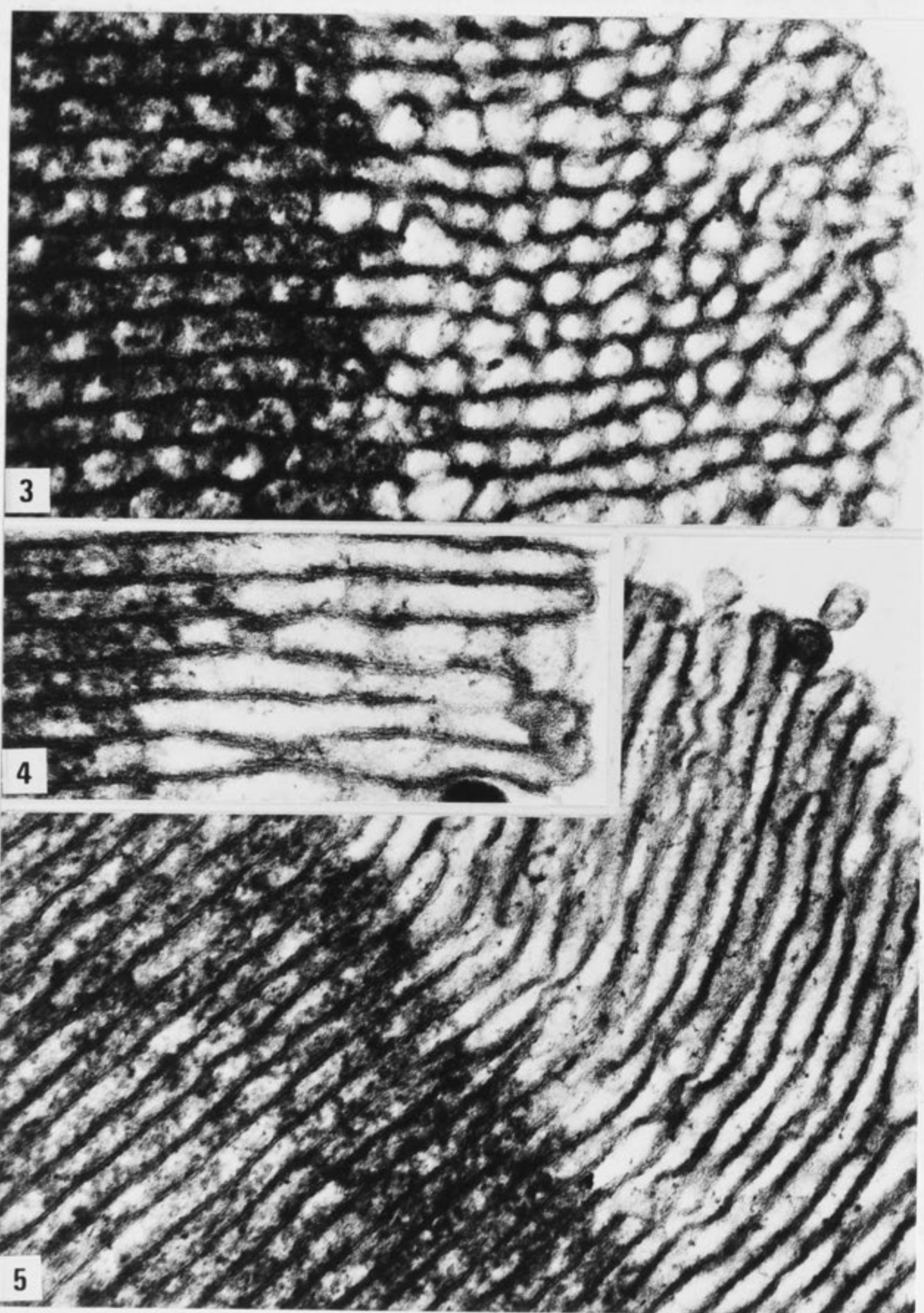
The distal microvillar regions destined for shedding form a well-defined zone in sections of rhabdoms which have been conventionally fixed for electron microscopy (figure 2). The shedding zone is produced by the gradual transformation of the tips of the microvilli throughout the day. In Ptilogyna, membrane is shed at some point between dawn and noon, to leave microvilli that either lack electron-lucent tips, or that possess short transformed regions (Williams & Blest 1980). Flies left in the dark for up to 72 h continue to assemble microvillar membrane and the return of such flies to the light provokes massive shedding. A small number of observations of Leptotarsus suggests that the control of turnover is different: flies held in the dark for long periods do not assemble an excess of rhabdomeral membrane, and fail to exhibit exaggerated shedding when returned to the light. A comparative study of flies identified to

species is needed. For the present purposes, both genera were sampled either after being held in darkness overnight until dissection during the morning, or some 3-4 h after dawn under a normal daily cycle of illumination, in order to obtain microvilli in a range of states.

The microvillar cytoskeleton

Our initial attempts to stabilise the cytoskeleton followed the procedures given by Blest et al. (1982), but the amount of EGTA in the stabilisation buffer was increased from 2 to 10 mM, and of Mg^{2+} from 2 to 15 mM. These treatments gave erratic and fragmentary preservation of the cytoskeleton, to reveal that the microvillar tips of the shedding zone are empty of contents (figures 3, 4 and 5) and that some material within the microvillar lumen had, at least, been immobilised by the procedure, but the overall picture was far from satisfactory. It was only marginally improved by using hypotonic buffers which caused a proportion of microvilli to swell slightly (figure 6). No additional improvement was yielded by combining the protease inhibitor TLCK with PMSF, and little difference could be observed between retinae supplied with 15 mM Mg^{2+} as the divalent cation and those given 15 mM Ba^{2+} . It is likely that the very large sizes of tipulid rhabdoms compared to those of blowflies make the satisfactory removal of Ca^{2+} by chelation impossible and the penetration of other reagents difficult.

Two sets of experiments were conducted with E-64 analogues. In one, retinae were treated during and after dissection with stabilisation buffer in which EGTA was replaced by 2 mM Ca^{2+} and to which was added 0.5 mM Ep-475 (E-64c). In the second series, 0.25 mM Ca^{2+} was combined with 1 mM Ep-459. Both series of trials were conducted



Figs. 3-5. Transitions between the stable regions of rhabdoms of Leptotarsus and their transformed shedding zones, after treatment with the basic stabilisation buffer and post-fixation mordanting in LMWT. There is a sharp demarcation between the shedding zones, whose microvillar segments are empty, and stable regions of the rhabdom where the microvilli contain an imperfectly-preserved cytoskeleton. Figs. 3, 4, X 120,000; Fig. 5, X 97,000.

with and without 1 mM PMSF, and in the case of the second series, both combinations were conducted with normal and hypotonic buffers. All were fixed in 2.5% glutaraldehyde without EGTA and with 10 mM Mg^{2+} , and were transferred to fixative solution containing EDTA after 30 m.

Both Ep-475 and Ep-459 are sufficient, by themselves, largely to preserve the axial filament in the presence of Ca^{2+} (figures 7-10). In our experiments, Ep-459 was the most effective, possibly as a consequence of better penetration. Figures 11 and 12 show the effect of incubating retinae in buffer containing 0.25 mM Ca^{2+} for 20 m without Ep-459. Without the inhibitor the microvillar contents were lysed and dispersed to become evenly distributed between the shedding zone and the basal region. In its presence, the microvillar tips remained empty and the basal region contained an organised cytoskeleton (figure 10). Even in the presence of high concentration of Mg^{2+} , the side-arms are poorly preserved.

No improvement was seen as a result of adding PMSF to the medium, and it was not necessary to employ hypotonic buffers. The dissolution of the cytoskeleton in the presence of Ca^{2+} , therefore, results solely from the action of a Ca^{2+} -activated neutral thiol protease. The difficulty of stabilising the cytoskeleton by conventional fixation implies a phenomenally rapid proteolytic action, and suggests that the protease is intimately associated with the filament it destroys.

Blest et al. (1982) believed that adding PMSF to their stabilisation buffer improved the resolution of the cytoskeleton in blowfly rhabdoms, but it is not clear that the concentrations of EGTA and Mg^{2+} which they used were inadequate. The possible implication of a second, serine protease in the blowfly system requires to be re-examined

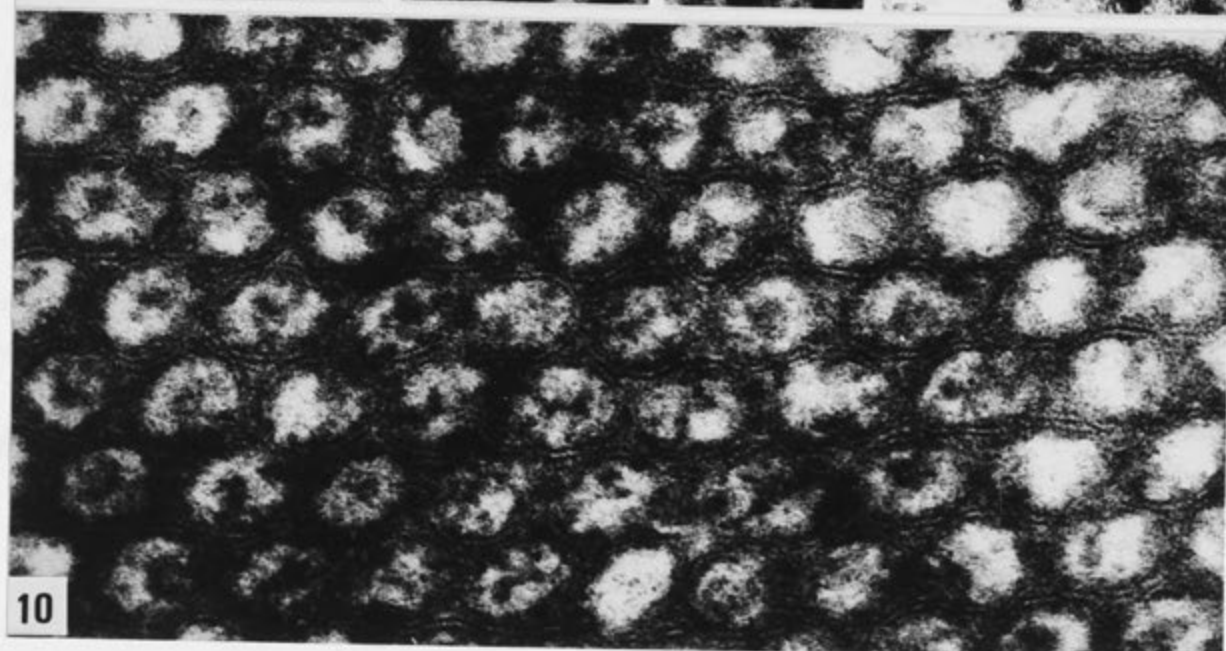
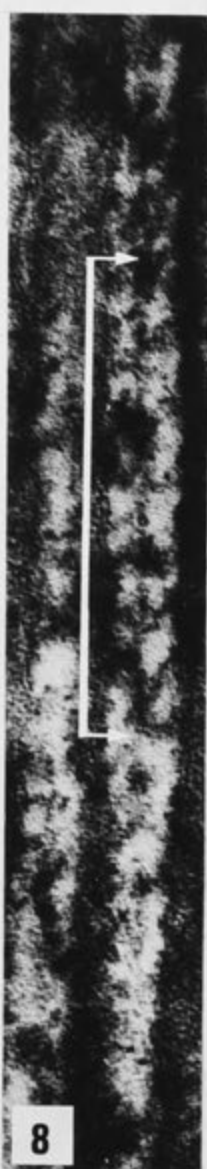
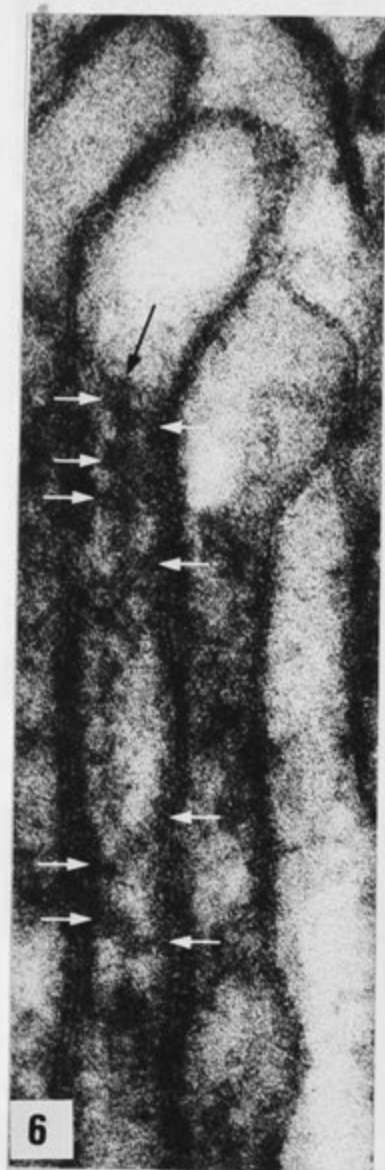
Fig. 6. (over page) Longitudinal section of microvillus tips after treatment with hypotonic stabilisation buffer containing 10 mM EGTA. The terminal segment of an axial filament is preserved in the central microvillus, and ends abruptly (black arrow) as the tip of the microvillus dilates. Lateral densities representing presumptive side-arms, or regions of close relationship between axial filament and plasma membrane are indicated by white arrows. X 170,000.

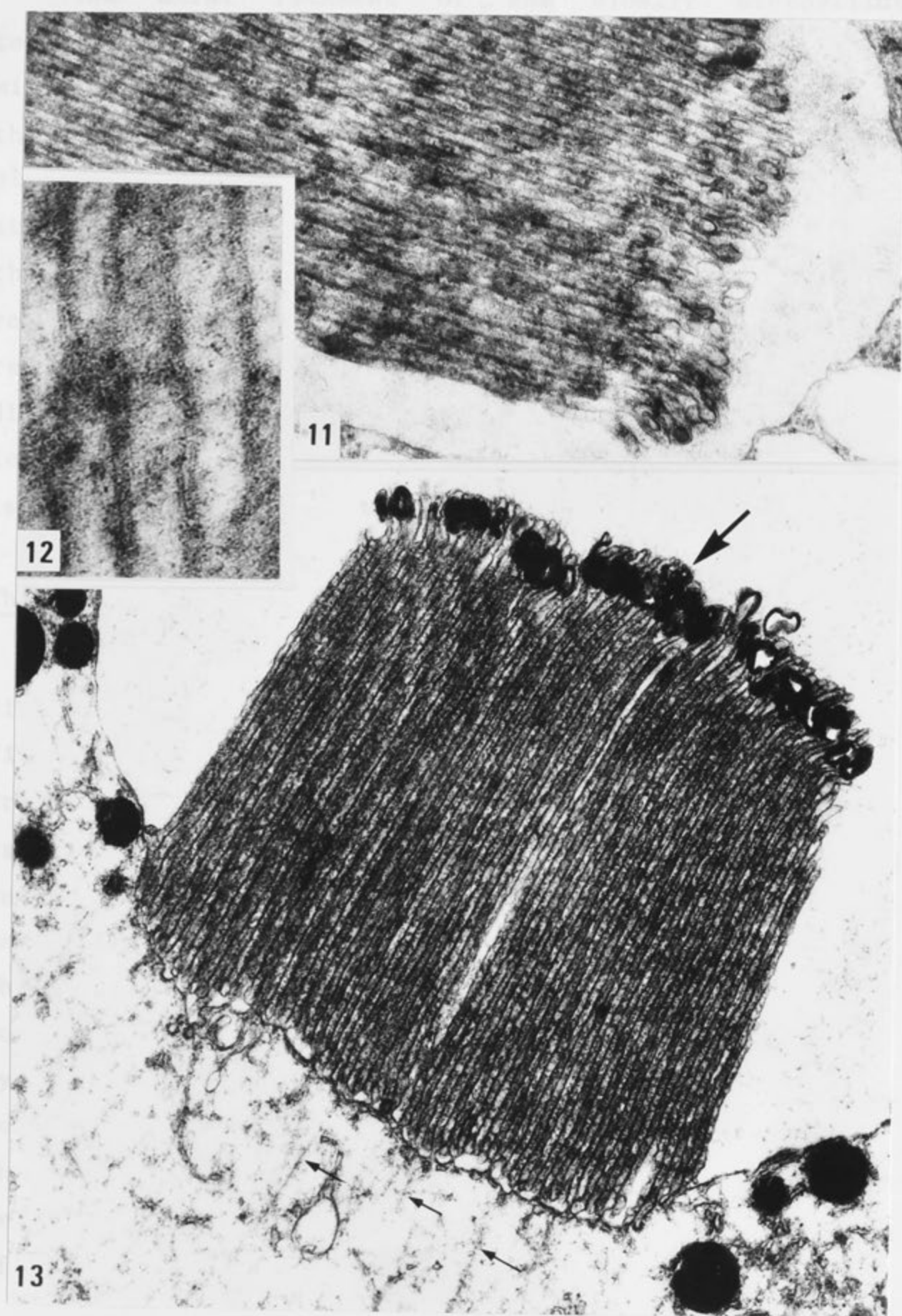
Figs. 7-9. (over page) Longitudinal sections of microvilli after treatment with stabilisation buffer containing 0.25 mM Ca^{++} and 1 mM Ep-459. Fig. 7 shows a continuous axial structure with ill-defined contacts with the plasma membrane; Fig. 8 shows irregular side-arms in the region bracketed between the two arrows; Fig. 9. shows small, circular densities (arrowed) which are often seen near the mid-line of the microvillus in longitudinal section, and which are assumed to be side-arms seen end on. X 182,000.

Fig. 10. (over page) Approximately transverse sections of microvilli after treatment with stabilisation buffer containing 0.25 mM Ca^{++} and 1 mM Ep-459. The transition to the shedding zone is at the far right, where the individual profiles are empty. Proximal regions of the microvilli have central densities which are fairly consistently present, linked by presumptive side-arms to the plasma membrane. X 170,000.

Figs. 11-12. (over 2 pages) Longitudinal sections of microvilli after treatment with buffer containing 0.25 mM Ca^{++} and 15 mM Mg^{++} , but without either EGTA or Ep-459. Microvilli are distorted, devoid of organised contents, and in Fig. 11 degraded material has dispersed, despite tannic acid mordanting. Fig. 11, X 37,000; Fig. 12, X 182,000.

Fig. 13. (over 2 pages) Transverse section of a rhabdom treated with stabilisation buffer containing 10 mM EGTA and 15 mM Mg^{++} , but without the use of EDTA in subsequent processing stages to chelate Mg^{++} after stabilisation of the side-arms of the cytoskeleton. The shedding zone (large arrows) has collapsed completely during osmication. Small arrows indicate radial filaments of the extra-rhabdomeral cytoskeleton. X 22,000.





Reversed cells

Blatt et al. (1982) proposed that destruction of the cell is required for the formation of the cell wall. This is the source of conventional fibroblasts.

in the context of experiments with E-64 analogues.

The axial filament of the blowfly microvillus terminates distally in a dense structure at the tip of the microvillus, termed the "cap" by Blest et al. (1982), and the neck of the microvillus contains an electron-dense plug which surrounds the end of the filament. Neither structure is found in the tipulid microvillus. Distally, the filament ends abruptly at the boundary of the empty region of the shedding zone (figure 6); the terminal region often seems especially electron dense and may differ from the proximal part of the filament. The longitudinal side-arm periodicity lies around 40 nm, and is variable, as it is in the blowfly.

The effects of divalent cations during fixation

White & Michaud (1981) noted that failure to remove divalent cations, including Mg^{2+} , from media used during fixation for electron microscopy produced membrane whorls from rhabdomeral membrane. Williams (1980b) earlier ascribed such effects to failure to remove Ca^{2+} , and suggested that they only occurred in regions from which membrane was about to be shed in the course of turnover. The prolonged tannic acid treatments used in the present work exacerbate these effects, and confirm Williams' hypothesis. Without EDTA to chelate Mg^{2+} , the shedding zone collapses completely (figure 13); in some sections, the basal region of the rhabdom is also damaged to a lesser degree in a pattern that resembles the distribution of pinocytotic vesicles (cf. figure 19).

Damaged cells

Blest et al. (1982) proposed that destruction of the cytoskeleton in the course of conventional fixation

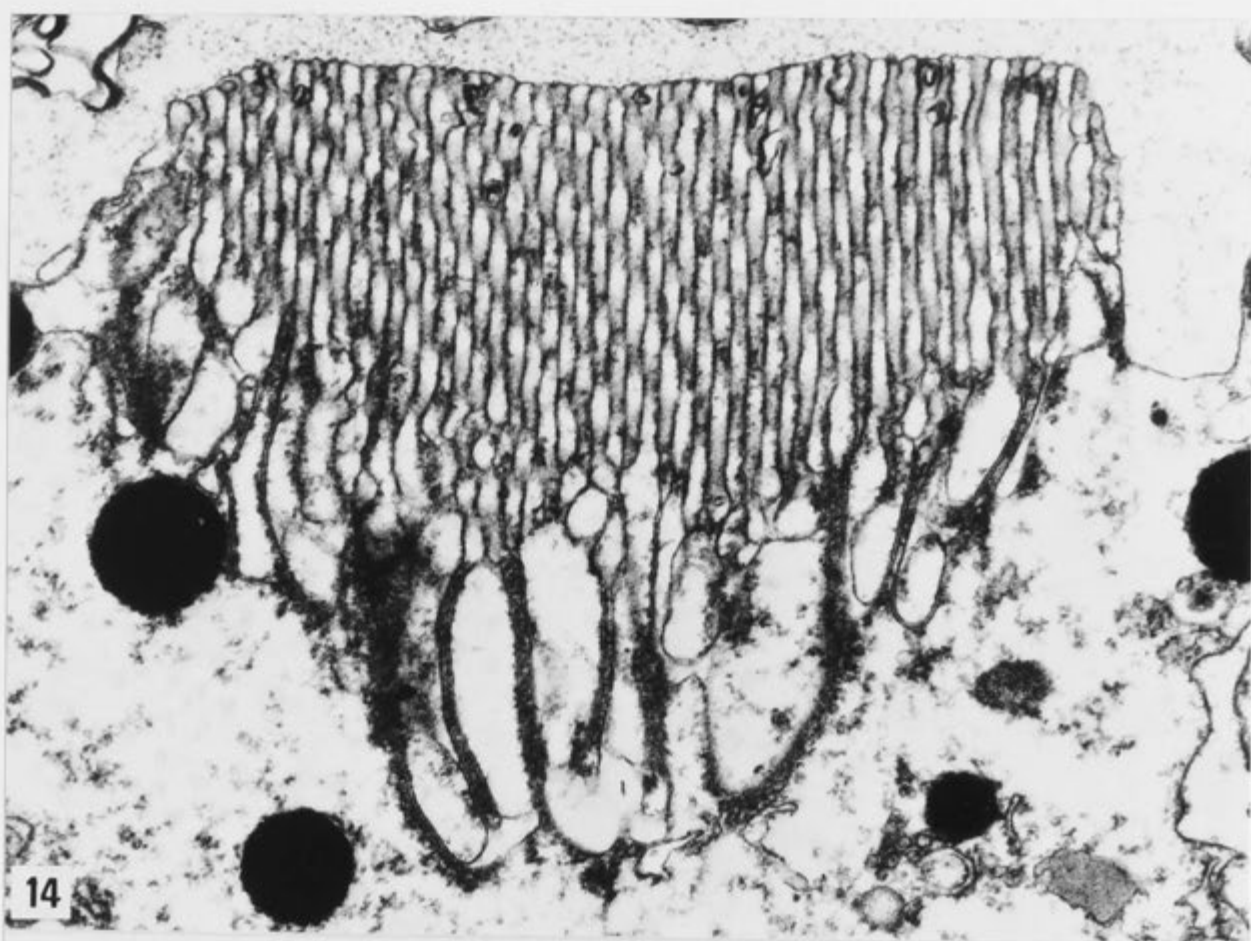
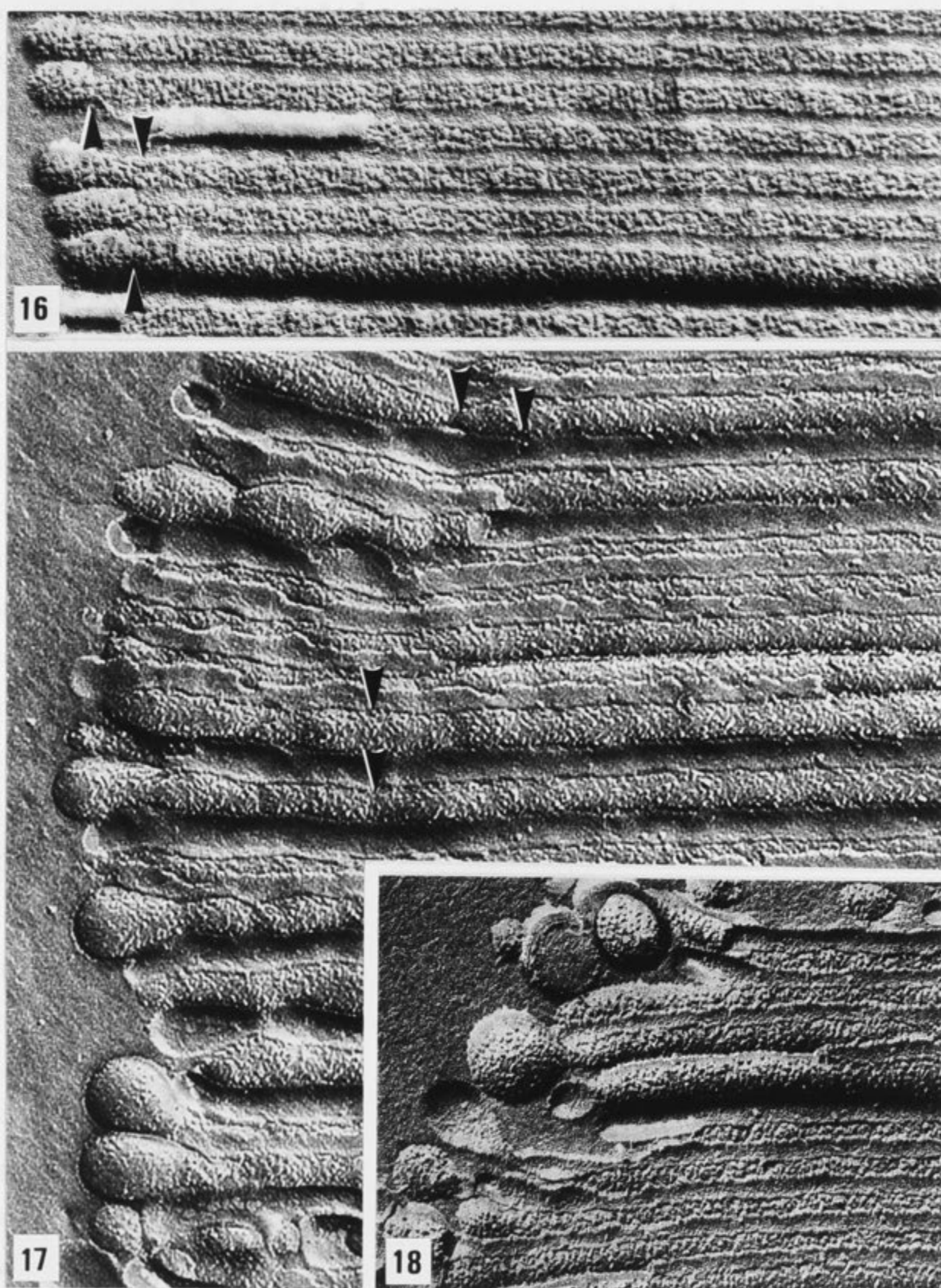


Fig. 14. Transverse section of a rhabdom from R1-6 after injury to its axon during dissection in stabilisation buffer containing 0.25 mM Ca^{++} and 1 mM Ep-459. Microvilli are grossly swollen compared to those of the stabilised rhabdom of Fig. 13, and the organisation of the rhabdom has begun to disintegrate at the proximal ends of the microvilli, which are devoid of structured contents. X 22,000.

Fig. 15. Microvilli from a damaged rhabdom in longitudinal section. A microvesicle of internalised membrane (arrowhead) lies within a microvillus, remote from the shedding zone. In one microvillus a small length of axial filament has survived (small arrows). X 120,000.



Figs. 16-18. Freeze-etch replicas of shedding zones in three states. In Fig. 16, only the extreme tip of each microvillus is transformed; in Fig. 17 transformed segments are substantial; in Fig. 18, the shedding zone is vesiculating. Points at which transistions from stable to shedding zones are presumed to occur are indicated by arrowheads. Figs. 16-17, X 92,000; Fig. 18, X 78,000.

occurred because rapid depolarisation of the invertebrate photoreceptor consequent upon damage generates an influx of calcium ions; it was suggested that this would switch on any Ca^{2+} -activated protease associated with the cytoskeleton. The optic lobes and lamina of Leptotarsus can be detached from the retina by pulling them gently: the whole basement membrane is stripped away neatly from the retina, breaking all the receptor axons. When this is done immediately after a half head has been immersed in buffer containing Ep-459, all rhabdoms assume the appearance shown in figures 14 and 15. The microvilli are swollen, often distorted, empty of organised contents, and disintegrating in a characteristic manner at their bases. If the medulla is separated from the lamina by a cut, thus severing the axons of R7-8 alone, only the rhabdoms of those two cells are affected. This suggests that depolarisation induced by damage does, indeed, activate rapid proteolysis before the inhibitor has penetrated to the rhabdom.

Freeze-etch observations

Observations were made on replicas derived from retinæ in a variety of shedding states. Satisfactory replicas of tipulid rhabdoms were difficult to obtain, because of problems associated with their large size and the rugged fracture topography generated by the large pigment granules. Shedding zones in three states are shown in figures 16 to 18. In figure 16, shedding has been recently completed, and only the extreme tips of the microvilli have been re-transformed. Comparisons between replicas and sections show that the shedding zone starts at approximately the levels marked for exemplary microvilli by arrowheads. In figure 17, an extensive

fringe has been transformed, and the irregular microvillar profiles of the shedding zone contrast with the regularity of the untransformed segments at the right. The microvillar tips in figure 18 have started to vesiculate and detach. In all cases, the density of particles on the P-faces of transformed and untransformed regions appears to be essentially the same.

The extrarhabdomeral cytoskeleton

Radially disposed elements are often observed at the base of the rhabdom, most frequently in retinae which have been treated with E64 analogues. Some are visible in figure 13. They will not be discussed here other than to note that one type with a diameter of 8 to 10 nm and irregular projections appears similar to the axial filament of the microvillus, is aligned parallel to the microvilli, and is sometimes so disposed as to suggest that it may be in continuity with the axial cytoskeleton. We have seen similar components in retinae of blowflies and Drosophila, and they resemble radially-arranged fibrillar elements in the retinulae of toadbugs (Burton & Stockhammer 1969). Similar radial arrays have also been observed in Musca (K. Kirschfeld, personal communication). A second type consists of a loosely radial arrangement of microfibrils that have the ultrastructural appearance of actin filaments. Even after treatment with PMSF and Ep-459 or Ep-475 in combination, neither component is consistently seen outside the rhabdom, and there is no relationship between successful preservation of the microvillar cytoskeleton and the presence of the extrarhabdomeral elements.

DISCUSSION

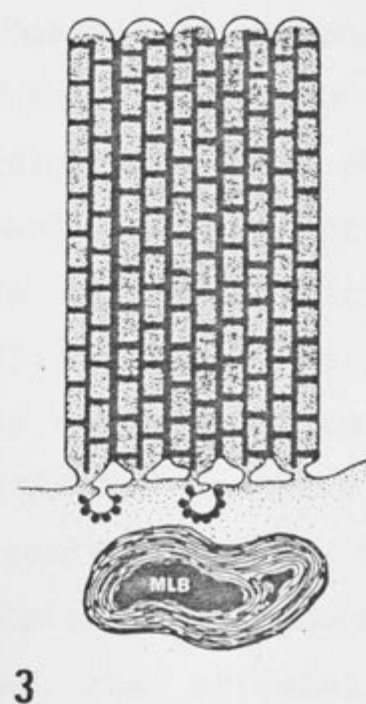
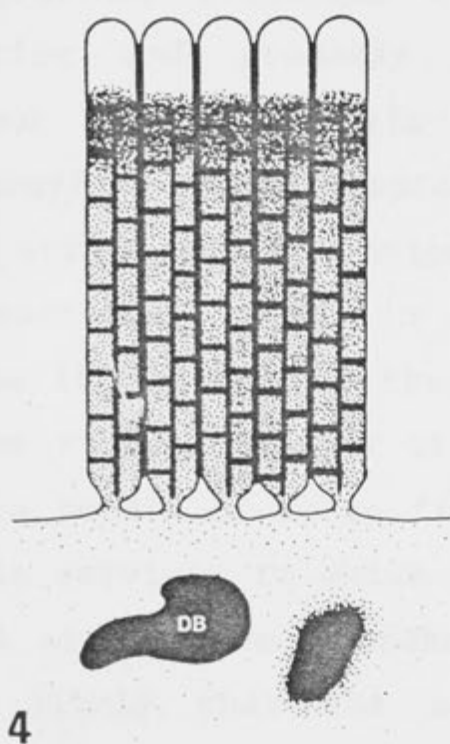
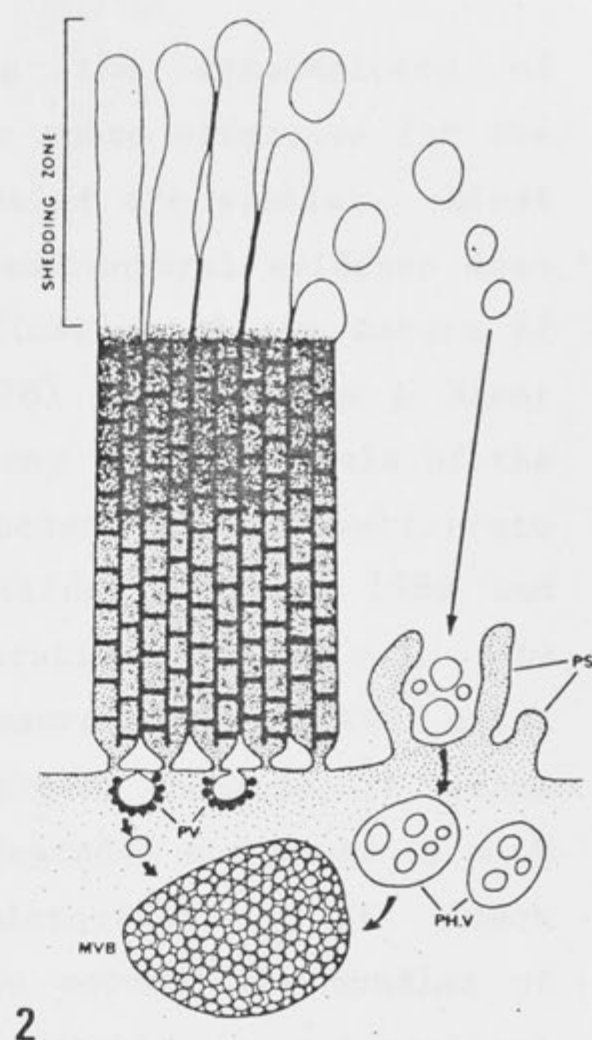
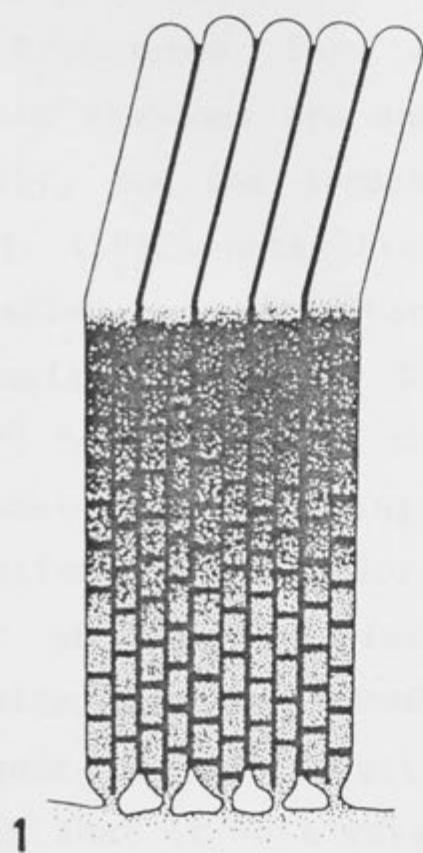
Although the microvillar cytoskeleton of tipulid flies has proved more difficult to preserve than that of blowfly photoreceptors, the same features have been revealed: an axial filament can be distinguished, usually in rather fragmentary form, linked by side-arms to the plasma membrane. When side-arms do not survive fixation, periodicities are often observed which probably represent their degradation products. In the tipulid microvillus, however, the cytoskeleton ends abruptly at a greater or lesser distance from the tip. The segment from which it is deleted corresponds to the region observed to be deformed in thin sections and freeze-etch replicas, but adjacent microvillar tips remain cemented together until shedding, presumably because the extracellular bridging material is still intact. Thus the internal cytoskeleton can be postulated to contribute to the rigidity of the microvillus and therefore to the regularity of the whole assembly. Nevertheless, substantial lengths of microvillus from which the cytoskeleton has been deleted must remain intact throughout the day; while deletion precedes shedding, and appears to define the limit of what will be shed, it cannot itself be responsible for abscission. Cytoskeletal deletion may, however, underlie the vulnerability of shedding zones to divalent cations during fixation, which the present study incidentally confirms.

The shedding of microvillar membrane is known to be a daily event (Williams & Blest 1980). It follows that as soon as a segment of microvillus has been shed, a new shedding zone is created by the withdrawal of material from the microvillus tip that remains. Much of this

material must consist of the cytoskeleton. A postulated sequence of events which combines our inferences about the behaviour of the cytoskeleton with the data of Williams & Blest (1980) is given in figure 19.

Transformation of local regions of microvilli by cytoskeletal deletion implies that the control of the microvillar infrastructure is complex. Blest et al. (1982) inferred that the cytoskeleton has an associated Ca^{2+} -activated protease with a calmodulin requirement as a minimal explanation of their pharmacological observations. The effectiveness of Ep-459 and Ep-475 in protecting the tipulid cytoskeleton confirms the presence of a Ca^{2+} -activated protease, for these analogues are specific inhibitors of thiol proteases, including vertebrate neurofilament proteases, thiol cathepsins and papain, while having no activity against either non-thiol proteases (trypsin, α -chymotrypsin, pepsin, elastase, bacterial collagenase) or non-proteolytic thiol enzymes (e.g. procine lactate dehydrogenase, bacterial glyceraldehyde . 3-phosphate dehydrogenase) (Tamai et al. 1981; K. Hanada, personal communication). The vulnerability of the cytoskeleton to Ca^{2+} in pretreatment solutions and fixatives implies that the protease is present throughout the length of the microvillus; either a second mode of activation exists, or deletion is mediated through a different enzymatic system. The results of preliminary experiments in which tipulid retinae were incubated in media combining the calmodulin blocking agent trifluoperazine with calcium were ambiguous, although the same media stabilise the cytoskeleton in blowfly rhabdoms (Blest et al. 1982). A role for calmodulin in the control of the cytoskeleton in tipulid rhabdoms is questionable, and in the case of the blowfly microvillus requires more

Fig. 19. (over page) A diagram to summarise the events inferred to take place in the course of the daily cycle of photoreceptor membrane turnover in Ptilogyna; it combines data from the present paper with that of Williams and Blest (1980). 1. Before dawn, the shedding zone is deep and composed of empty microvillar tips that are still adherent to each other. Near them, the stable region is particularly electron dense because it contains material transported from the shedding zone. 2. After dawn, membrane is shed from the microvilli by two routes; the microvillar segments in the shedding zone separate, vesiculate and drop off, and the membranous detritus is sequestered by pseudopodia of the receptor (PS), retrieved into phagocytotic vesicles (PH.V) and concentrated into large multivesicular bodies (MVB). The latter also receive pinocytotic vesicles derived from the microvillar bases (PV). 3. At the completion of shedding, the microvillar tips are untransformed, and the multivesicular bodies have started to degrade to multilamellar bodies (MLB). 4. By dusk, a new shedding zone has started to form, and most secondary lysosomes are degraded to dense bodies in the process of lysis (DB). The mode of addition of membrane in tipulids has not yet been analysed and is not therefore indicated in the scheme.



direct proof.

Procedures for stabilising the cytoskeleton of tipulid rhabdoms are analogous to those effective for the blowfly, and the structures revealed are similar. Blest et al. (1982) note that the ultrastructural evidence does not allow a satisfactory identification of the nature of the axial filament. Saibil (1978) and Horridge & Blest (1980) have suggested that actin may form the basis of the cytoskeleton, following the precedent of the vertebrate intestinal brush-border (cf. Horridge & Blest 1980 and Blest et al. 1982 for a comparative discussion). In tipulids, as in blowflies, measurements of the axial filament are difficult to interpret because it seems likely that it is always partly degraded and there is much adherent material. The microvilli of leech photoreceptors are wide enough to accommodate bundles of longitudinal filaments which are stable to conventional fixation and probably consist of actin (illustrated without comment by Walz 1979). During the morphogenesis of crayfish photoreceptor microvilli, a similar bundle, also stable to conventional fixation, has been observed; it vanishes later in development as the primordial microvilli narrow to the diameters characteristic of the mature rhabdom (Hafner et al. 1982). While it is possible that a bundle of actin filaments is thinned out to yield a single survivor to which a Ca^{2+} -activated protease is then added so that the cytoskeleton becomes unstable, it seems more likely that the actin skeleton is replaced by a neurofilament. The behaviour of the cytoskeleton to divalent cations (Blest et al. 1982) and the presence of an associated Ca^{2+} -activated neutral thiol protease is consonant with recent findings on invertebrate neurofilaments (Eagles et al. 1981; P.A.M. Eagles,

personal communication), although the rate of proteolysis is remarkable.

It is clear that the microvillar segments forming the shedding zones of tipulid rhabdoms are void of contents in life. This conclusion is reinforced by a comparison between retinae incubated in Ca^{2+} -containing buffers with and without the addition of Ep-459. In the presence of the inhibitor, the microvillar infrastructure is stabilised, and the transformed region remains empty. Incubated with Ca^{2+} alone, the cytoskeleton is lysed, and the products dispersed throughout the microvilli so that the shedding zone differs little in electron density from the basal segments. A further consequence is the implication that each microvillus possesses a system that transports mobilised materials towards its base.

An important question concerns the state of the membrane proteins and phospholipids in the transformed segments of the shedding zone. The freeze-etch technique does not distinguish between rhodopsin and metarhodopsin, nor does it reveal anything about the phospholipid components of the membrane. Thus, the present experiments do not allow us to say that the instability of the transformed region of the microvillus arises solely from the absence of the cytoskeleton; alterations to membrane components could also contribute to changes in its conformation (Kirkpatrick 1979). However, after incubation in 0.25 mM Ca^{2+} alone, all regions of the microvilli tend to an irregular and distorted appearance, consistent with a mechanical role for the internal cytoskeleton. This would not necessarily require the cytoskeleton to be static, and it is not impossible that it will prove to be in a state of flux in the living cell.

The demonstration of a microvillar cytoskeleton has

more general significance. For example, strong illumination causes the microvillar organisation of some rhabdoms to break down; light of defined wavelengths can be used selectively to destroy particular receptors in an ommatidium while sparing others, so that the spectral characteristics of the vulnerable cells can be inferred (e.g. Gribakin 1975; Welsch 1977; Langer et al. 1979). Analogous selective effects have been described when compound eyes are fixed for electron microscopy in aldehyde solutions of varied pH (Menzel & Blakers 1975). Authors have been tempted to ascribe these results of stress or hyperillumination to unspecified properties of the transductive membranes themselves, whilst admitting that a satisfactory explanation eludes them. The collapse of a supportive cytoskeleton in the face of excessive calcium fluxes mediating their effects through proteolysis is a more attractive basis for speculation, for it can be tested by simple experiments.

All rhabdoms fixed by conventional methods for light or electron microscopy are clearly left in a partly degraded state. Thus, the cumulative results of experiments in which rhabdoms are labelled by autoradiography (Perrelet 1972; Hafner & Bok 1977; Krauhs et al. 1978; Stein et al. 1979) are subject to two major difficulties of interpretation: (i) If cytoskeletal components turn over and are labelled by ^3H -leucine, then after fixation they will be broken down and topographically redistributed within each microvillus. Such a dispersal might account for the puzzling observation that after label has been injected, rhabdoms seem rapidly to be labelled throughout their depth. (ii) If proteolysis reduces the cytoskeleton to fragments of sufficiently small size, labelled cytoskeletal proteins

might elude detection on gels, and allow the conclusion of Stein et al. (1979) that the bulk of ^3H -leucine is incorporated into rhodopsin at the end of short-term labelling experiments.

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